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DEPARTMENT OF NUTRITIONAL PHYSIOLOGY AND FEEDING
ΕΡΓΑΣΤΗΡΙΟ ΦΥΣΙΟΛΟΓΙΑΣ ΘΡΕΨΕΩΣ ΚΑΙ ΔΙΑΤΡΟΦΗΣ

Ph.D. Thesis
ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

THE ANTIOXIDANT EFFECT OF DIETARY MICRO ALGAE SUPPLEMENTATION ON MILK, BLOOD AND RUMEN OF DAIRY GOATS

ΕΠΙΔΡΑΣΗ ΤΗΣ ΑΝΤΙΟΞΕΙΔΩΤΙΚΗΣ ΙΚΑΝΟΤΗΤΑΣ ΤΩΝ ΜΙΚΡΟΦΥΚΩΝ ΣΤΟ ΓΑΛΑ, ΤΟ ΑΙΜΑ ΚΑΙ ΤΗ ΜΕΓΑΛΗ ΚΟΙΛΙΑ ΜΕΤΑ ΤΗΝ ΕΝΣΩΜΑΤΩΣΗ ΤΟΥΣ ΣΤΟ ΣΙΤΗΡΕΣΙΟ ΑΙΓΩΝ

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Mahmoud Abdullah

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List of Abbreviations

ADF	Acid Detergent Fiber	**LCFA**	Long Chain Fatty Acid
AI	Atherogenicity Index	**LPO**	Lactoperoxidase
ALG	Algae Group with *Chlorella*	**MCFA**	Medium Chain Fatty Acids
As	Arsenic	**Mg**	Magnesium
BHA	Butylated Hydroxyanisole	**Mn**	Manganese
BHT	Butylated Hydroxytoluene	**Mo**	Molybdenum
Bp	Base Pair	**MUFA**	Mono Unsaturated Fatty Acids
C. pyrenoidose	*Chlorella pyrenoidosa*	**NADPH**	Nicotinamide Adenine Dinucleotide Phosphate
C. vulgaris	*Chlorella vulgaris*	**NDF**	Neutral Detergent Fiber
CAT	Catalase	**Ni**	Nickel
CLA	Conjugated Linoleic Acid	**$O_2^{\cdot-}$**	Superoxide anion
Co	Cobalt	**OM**	Organic Matter
CON	Control group without *Chlorella*	**Pb**	Lead
CP	Crude Protein	**PUFA**	Polyunsaturated Fatty Acid
Cu	Copper	**Q-PCR**	Quantitative PCR
DTT	Dichlorodiphenyltrichloroethane	**ROS**	Reactive Oxygen Species
DGGE	Denaturing Gradient Gel Electrophoresis	**S/U**	Saturated/Unsaturated
DHA	Docosahexaenoic Acid	**Sb**	Stibium
DM	Dry Matter	**SCP**	Single Cell Protein
DNA	Deoxyribonucleic Acid	**SCFA**	Short Chain Fatty Acids
EE	Ether Extracts	**Se**	Selenium
EPA	Eicosapentaenoic Acid	**SFA**	Saturated Fatty Acid
FA	Fatty Acid	**SOD**	Superoxide Dismutase
Fe	Iron	**USFA**	Unsaturated Fatty Acids
GR	Glutathione Reductase	**VA**	Vaccenic Acid
GSH-Px	Glutathione Peroxidase	**Zn**	Zinc
H_2O_2	Hydrogen Peroxide		

Abstract

Greek Abstract

ΕΠΙΔΡΑΣΗ ΤΗΣ ΑΝΤΙΟΞΕΙΔΩΤΙΚΗΣ ΙΚΑΝΟΤΗΤΑΣ ΤΩΝ ΜΙΚΡΟΦΥΚΩΝ ΣΤΟ ΓΑΛΑ, ΤΟ ΑΙΜΑ ΚΑΙ ΤΗ ΜΕΓΑΛΗ ΚΟΙΛΙΑ ΜΕΤΑ ΤΗΝ ΕΝΣΩΜΑΤΩΣΗ ΤΟΥΣ ΣΤΟ ΣΙΤΗΡΕΣΙΟ ΑΙΓΩΝ

Περίληψη

Σκοπός της παρούσας διατριβής ήταν να μελετήσει δύο στελέχη πράσινων φυκιών του είδους *Chrorella* (*C. pyrenoidosa* και *C. vulgaris*) ως μία φυσική πηγή αντιοξειδωτικών σε αίγες γαλακτοπαραγωγής και να διερευνήσει την επίδρασή τους: στην ενζυμική δραστηριότητα αντιοξειδωτικών ενζύμων στο πλάσμα του αίματος και στο γάλα, στο προφίλ των λιπαρών οξέων στο πλάσμα του αίματος, στο γάλα και στο υγρό της μεγάλης κοιλίας, στην ενζυμική δραστικότητα των ενζύμων της μεγάλης κοιλίας και στην μικροβιακή κοινότητα της μεγάλης κοιλίας, μέσω qPCR. Η μελέτη αποτελείται από δύο κύρια πειράματα.

Το πρώτο πείραμα διεξήχθη, για 4 εβδομάδες, με δεκαέξι γαλακτοπαραγωγές αίγες φυλής Alpine οι οποίες κατανεμήθηκαν σε δύο ομάδες (CON = μάρτυρας και ALG = επέμβαση) των οκτώ αιγών. Η ομάδα CON έλαβε ένα σιτηρέσιο χωρίς μικροφύκη, ενώ στην ομάδα ALG, 10g *C. pyrenoidosa* / kg ενσωματώθηκαν στο προς χορήγηση σιτηρέσιο. Η σίτιση των αιγών βασίστηκε στις χονδροειδείς ζωοτροφές, άχυρο σίτου και σανό μηδικής και σε ένα μίγμα συμπυκνωμένων ζωοτροφών σύμφωνο με τις απαιτήσεις τους. Δείγματα γάλακτος συλλέχθηκαν σε εβδομαδιαία βάση για χημική ανάλυση. Δείγματα γάλακτος και αίματος συλλέχθηκαν επίσης ανά περίοδο δύο εβδομάδων για τον προσδιορισμό της δραστικότητας των ενζύμων CAT, LPO, GST, GSH-Px, GR και SOD, καθώς και του προφίλ των λιπαρών οξέων. Την τελευταία ημέρα του πειράματος, συλλέχθηκαν δείγματα υγρού μεγάλης κοιλίας, με έναν στομαχικό σωλήνα, 3 ώρες μετά την πρωινή διατροφή και καταψύχθηκαν στους -80^{o}C για ανάλυση των λιπαρών οξέων των προστομάχων.

Τα αποτελέσματα έδειξαν ότι η συμπλήρωση της διατροφής των αιγών με *C. pyrenoidosa* παρουσίασε: **α)** αυξημένη ενεργότητα (U/ml) στα ένζυμα GR ($P<0,05$) και SOD ($P<0,01$), αυξημένη ($P<0.01$) συγκέντρωση πρωτεΐνης (σε mg/ml) στο πλάσμα του αίματος, και μειωμένη ($P<0,01$) δραστικότητα του ενζύμου GSH-Pχ (σε U/ml) στο γάλα σε σύγκριση με εκείνα της ομάδας CON. **β)** καμία επίδραση στην απόδοση και τη χημική σύνθεση του γάλακτος, ενώ υπήρξε μια στατιστικώς σημαντική αύξηση ($P<0,05$) της συγκέντρωσης των C18:1 trans και μείωση ($P>0,05$) της συγκέντρωσης των μικρής αλύσου κορεσμένων λιπαρών οξέων **γ)** αύξηση ($P< 0,05$) της συσσώρευσης του C18:0 και μείωση των ενδιάμεσων μορφών , των MUFA_και του λόγου C14:1/C14:0 ($P< 0,05$), στο υγρό της μεγάλης κοιλίας και **δ)** μείωση

(P< 0,05) των συγκεντρώσεων των ακόλουθων λιπαρών οξέων στο πλάσμα του αίματος: C18:2η-6 trans, C18:3n-6, C20:1, C21:0, C20:2 και C20:4.

Το δεύτερο πείραμα διεξήχθη με δεκαέξι αίγες φυλής Alpine που κατανεμήθηκαν σε δύο ομάδες (CON = μάρτυρας και ALG = επέμβαση) των οκτώ αίγων, για 4 εβδομάδες. Η ομάδα CON έλαβε μία δίαιτα χωρίς μικροφύκη ενώ στην ομάδα ALG, 10g *C. vulgaris*/kg ενσωματώθηκαν στο σιτηρέσιο. Η διατροφή των αιγών βασίστηκε σε χόρτο ψυχανθών, σανό μηδικής ως πηγή χονδροειδών ζωοτροφών, και ένα μίγμα συμπυκνωμένων ζωοτροφών σύμφωνο με τις απαιτήσεις τους. Δείγματα γάλακτος συλλέχθηκαν εβδομαδιαίως για χημική ανάλυση. Δείγματα αίματος και γάλακτος συλλέχθηκαν επίσης κάθε δύο εβδομάδες για να προσδιοριστεί η δραστικότητα των ενζύμων CAT, LPO, GST, GSH-Px, GR, SOD καθώς και το προφίλ των λιπαρών οξέων. Την τελευταία ημέρα του πειράματος, συλλέχθηκε υγρό μεγάλης κοιλίας, με ένα σωλήνα, 3 ώρες μετά την πρωινή διατροφή, το οποίο μετά από διήθηση καταψύχθηκε στους -80° C. Μέρος του υγρού κρατήθηκε χωρίς διήθηση και καταψύχθηκε στους -80 ° C για ανάλυση q-PCR.

Τα αποτελέσματα έδειξαν ότι η συμπλήρωση της διατροφής των αιγών με 10g/kg *C. vulgaris* παρουσίασε **α)** αυξημένη ενεργότητα (U/ml) στα ένζυμα GR (P<0,05), SOD (P<0,01), GST (P<0,001), GSH-Px (P<0,05) και αυξημένη (P<0,01) συγκέντρωση πρωτεΐνης (σε mg/ml) στο πλάσμα του αίματος, αυξημένη (P<0,001) δραστικότητα του ενζύμου LPO (σε U/ml) στο γάλα, σε σύγκριση με την ομάδα CON. **β)** καμία επίδραση στην απόδοση και τη χημική σύνθεση του γάλακτος **γ)** αύξηση της ατελούς βιοϋδρογόνωσης των C18:1 *trans* και C18:2n-6*trans* και αύξηση (P<0.001) των LCFA, AI, και του λόγου K/A λιπαρών οξέων στο γάλα Αυτή η συσσώρευση της υδρογόνωσης ενδιάμεσων των C18*trans* λιπαρών οξέων, συνδέθηκε με μείωση στην απόδοση των C18*cis* λιπαρών οξέων όπως C18:1*cis*-9 και C18:2η-6*cis* καθώς επίσης και με τη μείωση των λιπαρών οξέων που προέρχονται από *de novo* σύνθεση (<C16: 0). **δ)** μείωση των CLA (P<0,05), VA (P<0.001), και C18:1*trans*-10 (P<0.001) και αύξηση (P<0.05) του λόγου C18:1/C18:0 στο γάλα της ομάδας ALG σε σύγκριση με εκείνες της CON. **ε)** μείωση (P<0.05) των C14:0, C17:0, C20:1, και αύξηση (P<0,05) των C18: 0, C18:3n-6 και 18:2η-6*trans* (P>0,05)στο πλάσμα της ομάδας ALG σε σύγκριση την ομάδα CON. Η ίδια τάση παρατηρήθηκε και στις ενδιάμεσες μορφές ακόρεστων C18 στο πλάσμα και το γάλα. **στ)** βελτίωση της δραστικότητας της πρωτεάσης (P<0,01) και μείωση της δραστικότητας κελλουλάσης (P<0,01) στο υγρό της μεγάλης κοιλίας της ομάδας ALG σε σύγκριση με εκείνα της ομάδας CON. **ζ)** στατιστικώς σημαντική αύξηση (P<0,05) των *Butyrivibrio fibrosolvens*, αύξηση των *Ruminococcus flavefaciens, Clostridium sticklandii*, των *Μεθανογενών* και των πρωτόζωων και μείωση των *Ruminococcus albus* σε δείγματα του υγρού της μεγάλης κοιλία της ομάδας ALG σε σύγκριση με εκείνες της ομάδας CON.

Από τα αποτελέσματα αυτά της δραστικότητας των αντιοξειδωτικών ενζύμων και του προφίλ των λιπαρών οξέων του γάλακτος, συμπεραίνεται ότι η συμπλήρωση της διατροφής των

αιγών με δύο στελέχη της *Chlorella* οδηγεί σε σημαντική αύξηση των αντιοξειδωτικών δράσεων των ενζύμων στο πλάσμα του αίματος, αλλά η απόδοση της μεταφοράς στο γάλα είναι χαμηλή. Επίσης, η *Chlorella* και στα δύο πειράματα μείωσε την παραγωγή των ΛΟ που προέρχονται από *de novo* σύνθεση και αύξησε τα C18:1 *trans*. Η αύξηση του C18:1 *trans* θα μπορούσε να παίξει ένα προστατευτικό ρόλο για τα βακτήρια. Στις αίγες γαλακτοπαραγωγής η μείωση αυτών των λιπαρών οξέων είναι πολύ σημαντική και έχει συσχετιστεί θετικά με την ιδιαίτερη γεύση του αίγειου γάλακτος. Επίσης η προσθήκη *C. Vulgaris* βελτίωσε το μικροβιακό πληθυσμό και την ενζυμική δραστικότητα των ενζύμων (α-αμυλάση, πρωτεάση και λιπάση) και μείωσε τη δραστικότητα της σελουλάσης του υγρού της μεγάλης κοιλίας.

Λέξεις κλειδιά: *Chlorella,* πλάσμα, γάλα, αντιοξειδωτικά ένζυμα, το προφίλ των λιπαρών οξέων, μεγάλη κοιλία, q-PCR, αίγες.

THE ANTIOXIDANT EFFECT OF DIETARY MICRO ALGAE SUPPLEMENTATION ON MILK, BLOOD AND RUMEN OF DAIRY GOATS

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Abstract

The aim of the present study was to investigate two strains of green algae *Chlorella* (*C. pyrenoidosa and C. vulgaris*) as a natural antioxidants source for dairy goats and to study their effects on: blood plasma and milk antioxidant enzymes activities, rumen fermentation characteristics, blood plasma and milk fatty acid profile, rumen enzymes activities and q-PCR for rumen microbial community. The study included two main experiments as follow:

The first experiment was carried out with sixteen dairy crossbred Alpine goats allocated into two groups (CON= control and ALG= treated) of eight goats each for 4 weeks. The CON group received a diet without micro algae, whereas in the ALG group, 10g *C. pyrenoidosa* /kg were incorporated into concentrates. Goat's feeding was based on wheat straw and alfalfa hay as roughage source, and a concentrate mixture according to their requirements. Milk samples were collected weekly for chemical analysis. Blood and milk samples were also taken every two weeks to determine the activities of CAT, LPO, GST, GSH-Px, GR and SOD enzymes and the fatty acid profile. On the last day of the experiment, rumen samples were collected by a stomach tube 3 hrs after morning feeding and frozen at -80 ^{0}C for rumen fatty acids analysis.

The results showed that the supplementation of the goat's diet with *C. pyrenoidosa* had: a. increased the GR ($P<0.05$), SOD ($P<0.01$) activities (in U/ml), and ($P<0.01$) protein concentration (mg/ml) in blood plasma, and decreased ($P<0.01$) milk GSH-Px (U/ml) activity, b. no effect on milk yield and milk chemical composition, while there was an increase ($P<0.05$) in C18:1 trans and a decrease ($P>0.05$) in SCFA concentrations, c. an increase of accumulation of C18:0 concentration and a decrease in the C18 intermediates, ($P<0.05$) MUFA concentrations and ($P<0.05$) C14:1/C14:0 ratio in rumen, and d. decreased ($P<0.05$) C18:2n-6 trans, C18:3n-6, C20:1, C21:0, C20:2, and C20:4 concentrations in blood plasma.

The second experiment was carried out with sixteen dairy crossbred Alpine goats which were allocated into two groups (CON=control and ALG=treated) of eight goats each for 4 weeks. The CON group received a diet without micro algae whereas in the ALG group, 10g *C. vulgaris* /kg were incorporated in the concentrates. Goat's nutrition was based on Onobrychis viciifolia hay and alfalfa hay as a roughage source, and a concentrate according to their requirements. Milk samples were collected weekly for chemical analysis. Blood and milk samples were also collected every two weeks to determine the activities of CAT, LPO, GST, GSH-Px, GR and SOD enzymes and fatty acid profile. On the last day of the experiment, rumen liquid was collected by a stomach tube 3 hrs after morning feeding for rumen enzymes analysis and fatty acid profile. Part of the rumen fluid was kept at -80 ºC for q-PCR analysis.

The supplementation of goat diets with 10g *C. vulgaris/* kg concentrates had also no effect on milk yield and milk chemical composition but it had: a. increased in the GST ($P<0.001$), GR ($P<0.05$), GSH-Px ($P<0.05$), SOD ($P<0.01$) activities (U/ml), and protein concentration ($P<0.01$) in blood plasma and increased ($P<0.001$) LPO activity (U/ml) in milk. b. increased in accumulate an incomplete biohydrogenation of C18:1trans and C18:3n-3 concentrations and result in a strong accumulation of VA and C18:1 trans-10 and C18:2n-6 concentrations in rumen. The linolenic acid (C18:3n-3) in the rumen leads to the formation of VA and CLA, c. increased incomplete biohydrogenation of C18:1 trans and C18:2n-6trans concentrations. This accumulation of hydrogenation intermediate of C18 trans FA's associated with decreased in the concentrations yield of C18cis FA's such as C18:1 cis-9 and C18:2n-6cis and also decreased FA originating from synthesized *de novo* (<C16:0) and increased ($P<0.001$) LCFA concentration, AI, and S/U ratio in milk, d. decreased milk CLA, VA, and C18:1 trans-10 concentrations ($P<0.05$, $P<0.001$, and $P<0.001$ respectively) and increased ($P<0.05$) *C*18:1/*C*18:0 ratio. e. decreased plasma ($P<0.05$) C14:0, C17:0, C20:1 concentrations and increased ($P<0.05$) C18:0, C18:3n-6 and ($P>0.05$) 18:2n-6 trans concentrations. The same trend was observed in unsaturated C18 intermediate in plasma and milk, f. improved protease activity ($P < 0.01$) and reduced cellulase activity ($P< 0.01$) in rumen samples, and g. significantly increased ($P<0.05$) in the population of *Butyrivibrio fibrosolvens*, insgnificantly increased *Ruminococcus flavefaciens*, C*lostridium sticklandii*, Methanogens and protozoa and insignificant reduction of *Ruminococcus albus* to the total population in rumen samples.

From those results of the antioxidant enzyme activities and the milk fatty acid profile, it was concluded that the supplementation of goat's diet with two strains of *Chlorella* leads to significant increases of the antioxidant enzyme activities in blood plasma but not in milk. Also, the *Chlorella* in both experiments decreased the yield of FA originating from *de novo* synthesis and increased the C18:1 trans concentration. The increase in C18:1 trans production could play a protective role for bacteria. In conclusion, in dairy goats the decrease of concentrations these FA is very important and it has been positively related to "goaty" flavor in goat milk. Also, the adition of *C. vulgaris* improved the microbial population and the rumen enzymes activity (α-amylase, protease and lipase) and redused cellulase activity.

Key words: *Chlorella*, plasma, milk, antioxidant enzymes, fatty acid profile, rumen q-PCR, goats

Introduction

1 Introduction

Milk and dairy products are recognized as important sources of nutrients in human diets, providing energy, high quality protein, and essential minerals and vitamins (NRC, 1998; Demment and Allen, 2004). Milk fat is responsible for many of the sensory, physical, and manufacturing properties of dairy products (Kaylegain and Lindsay, 1995).

Micro algae are able to enhance the nutritional content of conventional food and feed preparation and hence to positively affect humans and animal health due to their original chemical composition, namely high protein content with balanced amino acids pattern, carotenoids, fatty acids, vitamins, polysaccharides, sterols, phycobilins and other antioxidants (Gouveia *et al*., 2008).

Macro algae (Madhusudan *et al*., 2011) or micro algae (Christaki *et al*., 2011; Hoa *et al*., 2011) have been used in animal diets, in order to enhance the nutritional value of its products. Micro algae are prokaryotic or eukaryotic photosynthetic microorganisms that produce carbohydrates, proteins and lipids as a result of photosynthesis (Richmond, 2004). The most biotechnologically relevant micro algae is the green algae (Chlorophycea) *Chlorella*, which is already widely commercialized and used, mainly as a nutritional supplement for humans and as animal feed additive (Gouveia *et al*., 2008).

Each *Chlorella* microorganism contains 28.9 g chlorophyll /kg; its proteins contain all the amino acids known to be essential for the nutrition of animals and human beings. Vitamins found in *Chlorella* include vitamin C, provitamin A (α-carotene), thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), niacin, pantothenic acid, folic acid, vitamin B_{12}, biotin, choline, vitamin K, lipoic acid, and inositol. Minerals in *Chlorella* include phosphorus, calcium, zinc, iodine, magnesium, iron, and copper. Steenblock (1987) reported that Chlorella extract contains *Chlorella* growth factor (CGF) along with malic acid (apple acid), fructose, lemon essence, and water. Some of these bioactive compounds have the capacity to scavenge the free radicals.

Due to the fact that micro algae have the above biological active substances, they have been used in human (Jose and Arley, 2013) and animal nutrition; rats (Vijayavel *et al*.,

2007; Su-Ching *et al*., 2012), poultry (Gouveia., 1996, Carrillo *et al*., 2008) , pigs (Sardi *et al*., 2006, Bichi *et al*., 2013), dairy cows (Lopex *et al*., 2013), dairy ewes (Papadopoulos *et al*., 2002, Toral *et al*., 2010 and Moreno- Indias *et al*., 2014).

Most of the already published studies have used micro algae with high fat content in order to modify the FA profile of milk and other animal products. However, to the best of our knowledge there is no other published work which has been examined the impact of micro algae which are rich in protein, antioxidant compounds, and low in fat on milk, blood and rumen parameters. Our study is focused on two strains of green algae *Chlorella; Chlorella pyrenoidosa* (*C. pyrenoidosa*) and *Chlorella vulgaris* (*C. vulgaris*) as a source of natural antioxidant for dairy goats and investigates the effects on: blood plasma and milk antioxidant enzymes activities; blood plasma and milk fatty acid profile; rumen enzymes activities and q-PCR in rumen microbial community.

STRUCTURE OF THE THESIS

The present thesis is divided into three parts;

Part A, Literature Review: provides background and review of literature about micro algae and their contribution to animal nutrition.

Part B, Experiments: presents the two experiments of the thesis which were carried out with two strains of *Chlorella.*

Part C, General Discussion and conclusion: gives a conclusive summary of the present findings, general conclusions and provides recommendations for future research.

Part A:
Literature Review

2 Literature Review

2.1 What are micro algae?

Algae are aquatic, photosynthetic microorganisms that appeared on earth about 3.5 billion years ago and are regarded as the first life form (Margulis, 1981). They are distinguished into multicellular organisms (macro algae) that can reach 60 m in length and single-celled organisms (micro algae or phytoplankton) with size from 0.2 to 2 mm (Harlin and Darley 1988). They are reproduced by simple division one or two times per day. They have no roots, stems, flowers or leaves, so they grow much faster than terrestrial plants and are characterized as the most productive plants in the world (Marshall, 2007).

Although the micro algae biotechnology is similar to conventional agriculture, micro algae have many advantages over terrestrial plants. They have higher productivity than traditional crops and can be grown in climatic conditions and regions where other crops cannot.

It has been estimated that more than 30,000 species of micro algae exist, but the chemical composition of only some hundreds of those has been studied and only a few are cultivated in significant (industrial) quantities (Gouveia *et al*., 2008).

2.2 Micro algae as a novel feedstuff

While the nutritional profile of micro algae vary considerably with the species used, a large majority of them are characterized by high protein, carbohydrates, and lipid contents that are comparable, if not superior, to conventional feedstuffs (Table 2.3.1.1).

Nowadays, consumers' demand for natural and healthy products is constantly increasing. Therefore, there is a great interest for novel functional foods. A strategy for producing such foods is through the modification of animal diets using bioactive feed supplements, such as macro algae (Madhusudan *et al*., 2011) or micro algae (Christaki *et al*., 2011; Hoa *et al*., 2011).

Micro algae are an enormous biological resource, representing one of the most promising sources for new products and applications (Pulz and Gross, 2004). They can be

used to enhance the nutritional value of food and animal feed, due to their well-balanced chemical composition. Moreover, they are cultivated as a source of highly valuable molecules and other biologically active compounds. The application of micro algae biomass and/or metabolites is an interesting and innovative approach for the development of healthier food products. Micro algae are able to enhance the nutritional content of conventional food and feed preparation and hence to positively affect humans and animals health due to their original chemical composition than traditional crops.

In spite of the relatively high crude protein content, many micro algae species may still show limited biological value for the proteinaceous biomass due to the presence of non-protein nitrogen that consists of nucleic acids, nitrogen-containing cell walls, and amines. As single cell proteins, micro algae are photosynthetic unicellular eukaryotes containing nucleic acids that may represent approximately 10% of the crude protein (Becker, 2004). Ruminal bacteria are effective in utilizing non-protein nitrogen to synthesize protein (McNaught and Owen, 1954).

2.3 Chemical composition and bioactive molecules in micro algae

As higher plants, the chemical composition of micro algae is not an intrinsic constant factor but varies over a wide range. Environmental factors, such as temperature, illumination, pH-value, mineral contents, CO_2 supply, or population density, growth phase and micro algae physiology, can greatly modify their chemical composition. Micro algae can biosynthesize, metabolize, accumulate and secrete a great diversity of primary and secondary metabolites, many of which are valuable substances with potential applications in the food, pharmaceutical and cosmetics industries (Yamaguchi, 1997).

Micro algae contain substances of high biological value, such as polyunsaturated fatty acids (PUFA), proteins, pigments, antioxidants, vitamins and minerals. Also, recent studies indicate that micro algae could be used to produce biofuels (Mata *et al*., 2010) and hydrogen (Dutta *et al*., 2005). Table 2.3.1.1 shows the nutrient composition (%) of conventional feedstuffs and edible micro algae.

2.3.1 Micro algae as a source of polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids, such as omega-3 series, i.e. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), derived from micro algae are important products, which nowadays are used in the food and feed industry (Pulz and Gross, 2004). These PUFA's cannot be synthesized by humans or animals, so they should be included in the daily diet (Simopoulos, 2002). Some species of micro algae can produce these FA's in quantities varying not only between different species, but also between different phases of development (Spolaore *et al*., 2006a).

The micro algae oil content varies widely and may reach even 70% of dry matter, whereas in most micro algae the content is between 20 and 50% (Mata *et al*., 2010). Some families of micro algae (*Schizochytrium sp.)* are rich in DHA (25%) and C22:5 (n-6) (12%), but poor in EPA (1%) (Barclay *et al*., 1998), while other families (*Chlorella*) are low in DHA (0.22-0.30%) and EPA (0.30-0.40%) (Ötleô and Pire, 2001). Table 2.3.1.2 shows fatty acid composition of different *Chlorella* species.

Table 2.3.1.1 Nutrient composition of conventional feedstuffs and edible micro algae (% dry matter).

***Source*[a]**	**Crude Protein**	**Carbohydrates**	**Lipids**	**Minerals**
Soybean	37	30	20	-
Corn	10	85	4	-
wheat	14	84	2	-
Chlorella vulgaris	51-58	12-17	14-22	5-10
Chlorella pyrenoidosa	57	10-17	2	5-10
Scenedesmus obliquus	50-56	10-17	12-14	-
Scenedesmus quadricauda	47	-	2	-
Spirulina platensis	46-50	8-14	4-9	-
Spirulina maxima	60-71	13-16	6-7	

[a] Adapted from (Phang, 1992; Becker, 1994)

Table 2.3.1.2 Fatty acid composition of different *Chlorella* species (% of total fatty acids) [a]

Fatty acid	Name	*C. pyrenoidosa*	*C. vulgaris*
6:0	**Caproic**	1.65	2.77
8:0	**Caprylic**	0.88	0.26
11:0	**Undecanoic**	0.14	1.39
11:1	**Undecenoic**	1.70	2.17
12:0	**Lauric**	1.02	0.87
12:1	**Lauroleic**	0.12	0.41
13:0	**Tridecanoic**	0.15	1.03
14:0	**Myristic**	0.31	0.69
15:0	**Pentadecanoic**	0.12	1.70
15:1	**Pentadecenoic**	0.14	3.53
16:0	**Palmitic**	17.22	14.42
16:1	**Palmitoleic**	3.67	4.04
16:2	**Hexadecadienoic**	2.43	5.34
16:3	**Hexadecatrienoic**	4.45	4.90
17:0	**Margaric**	0.20	0.12
17:1	**Heptadecenoic**	0.19	0.27
18:0	**Stearic**	2.55	1.57
18:1	**Oleic**	19.71	17.62
18:2 n-6	**Linoleic**	21.55	11.97
18:3 n-6	**Gamma linolenic**	0.51	Nd
18:3 n-3	**Alpha linolenic (ALA)**	13.81	15.79
20:0	**Arachidic**	Nd [b]	0.14
20:2 n-6	**Eicosadienoic**	0.39	Nd
20:3 n-8	**Dihomolinolenic**	Nd	Nd
20:5 n-3	**Eicosapentaenoic**	0.31	Nd
22:1	**Docosaenoic**	Nd	Nd
22:6 n-3	**Docosahexaenoic**	0.22	0.30
23:0	**Tricosanoic**	0.18	Nd
24:0	**Tetracosanoic**	Nd	0.22
24:1	**Tetracosenoic**	0.38	Nd
Others	**Tetracosenoic**	5.69	8.28

[a]Source: Ötleô and Pire (2001) [b] Nd= Not detected

2.3.2 Micro algae as a source of carbohydrates

Carbohydrates of micro algae can be found in the form of starch, cellulose, sugars, and other polysaccharides. As the overall digestibility of the carbohydrates of the tested micro algae is good, it seems to be no limitation in using dried micro algae as a whole. Whether carbohydrates are likely to cause any other problems like gastro-intestinal disturbance, flatulence or fluid retention, can be established by *in vivo* experiments only (Becker, 2004).

2.3.3 Micro algae as a source of protein

Since the early fifties intense efforts have been made to explore new alternate protein sources as food supplements, primarily in anticipation of a repeatedly predicted insufficient future protein supply. For these, i.e. yeasts, fungi, bacteria and micro algae, the name Single Cell Protein (SCP) was coined to describe the protein production from biomass, originating from different microbial sources (Becker, 2007).

Indeed, unlike other plants, most micro algae contain essential amino acids which cannot be synthesized in human or animal bodies (Gouveia *et al*., 2008). Becker (2007) states that the content of amino acids (lysine, methionine, tryptophan, threonine, valine, histidine and isoleucine) in some micro algae is comparable with that of egg or soybean. Table 2.3.3.1 shows the amino acids content (g / 100 g of protein) of *C. vulgaris* compared with that of soybean and hens' egg.

The high protein content of various micro algae species is one of the main reasons to consider them as an unconventional source of protein well illustrated by the great interest in micro algae as SCP during the 1950s (Soletto *et al*., 2005). As other bioactive compounds synthesized by micro algae, amino acids composition, especially the free amino acids, varies greatly between species as well as with growth conditions and growth phase (Borowitzka, 1988). Protein or amino acids may therefore be by-products of an algae process for the production of other fine chemicals, or with appropriate genetic enhancement, micro algae could produce desirable amino acids in sufficiently high concentrations (Borowitzka, 1988).

Table 2.3.3.1 Amino acids content (g / 100 g of protein) of *Chlorella vulgaris* compared with that of soybean and hens' egg

Amino acids	*C. vulgaris*[€]	Soybean*	Hen egg[€]
Alanine	7.8	5.0	-
Arginine	7.9	7.4	5.7
Cysteine	0.27	1.9	10.5
Aspartic acid	9.70	1.3	2.3
Glutamic acid	13.1	19.0	12.6
Isoleucine	5.1	-	3.0
Leucine	2.0	7.7	8.8
Lysine	5.2	6.4	7.0
Methionine	9.1	1.3	9.2
Phenylalanine	8.4	5.0	7.4
Proline	2.4	5.3	3.0
Serine	5.2	5.8	5.1
Threonine	6.0	4.0	5.0
Tryptophane	4.0	1.4	8.4
Tyrosine	3.9	3.7	4.1
Valine	2.4	5.3	1.1
Glycine	5.8	4.5	4.2
Histidine	7.8	2.6	2.4

Source: [€]Khatun *et al*. (1994) ; * WHO/FAO (1973)

2.3.4 Micro algae as a source of vitamins and minerals

Beside the high levels of protein, lipids and carbohydrates, micro algae also contain appreciable amounts of valuable vitamins and minerals (Table 2.3.4.1). Some algae species are known to rapidly accumulate heavy metals at concentrations higher than their surroundings (Becker, 2004), while others generate pathological metabolites that cause neuro-degenerative disorders (Jin *et al*., 2006).

Micro algae represent an important source of nearly all the important vitamins such as tocopherols, ascorbic acid, B1, B2, B6, B12, nicotinic acid, biotin and so on as well as macro minerals (Na, K, Ca and Mg) and micro minerals (Fe, Zn, Mn and Cu) (Fabregas and Herrero, 1990; Becker, 1994; Spolaore *et al*., 2006a). *Chlorella* is rich in P (1761.5 mg), Na (1346.4 mg), K (749.9 mg), Ca (593.7 mg), mg (344.3 mg), and Fe (259.1 mg). Other mineral contents include Mn (2.09 mg), Zn (1.19 mg), Se (0.07 mg), Cu (0.06 mg), and Cr (0.02 mg) (Oglu and Unal, 2003). Furthermore, administration of *Chlorella* prevents cadmium-induced toxicity and toxin-caused oxidative stress and cellular damage. *Chlorella vulgaris* administration maintains the renal cytoarchitecture against $HgCl_2$-caused oxidative stress and nephrotoxicity (Valdivia *et al*., 2011).

The vitamin content of in micro algae depends on the genotype, the stage in the growth cycle, the nutritional status of the micro algae, the light intensity (photosynthetic rate). The vitamin content is therefore amenable to manipulate by varying the culture conditions as well as by strain selection or genetic engineering. However, vitamins cell content changes with environmental factors, the harvesting treatment and the biomass drying methods (Borowitzka, 1988; Brown *et al*., 1999).

Table 2.3.4.1 Analytical data of minerals (mg/kg DM) in *Chlorella*.

Items	*Chlorella sp.*[a]
Ca	2250
P	11850
K	7546
Mg	2530
Fe	480
Cu	2.6
Co	0.20
Cd	0.03
Pb	0.69
Se	0.121
Mo	0.198
Zn	21

[a] Source: Bruno *et al*. (2012)

2.3.5 Micro algae as a source of pigments

One of the most obvious and arresting characteristic of the micro algae is their color. In general, each phylum has its own particular combination of pigments and an individual color. Aside chlorophylls, as the primary photosynthetic pigment, micro algae also form various accessory or secondary pigments, such as phycobiliproteins and a wide range of carotenoids. Therefore, micro algae are recognized as an excellent source of natural colorants and nutraceuticals and it is expected that they will surpass synthetics as well as other natural sources due to their sustainability of production and renewable nature (Dufossé *et al*., 2005).

Apart from chlorophyll, which is the major photosynthetic pigment, micro algae also contain phycobiliproteins and a wide variety of carotenoids. These substances act as antioxidants for the animal organisms rather than consume micro algae (Gouveia *et al*., 2008). Chlorophyll is contained in micro algae at 0.5–1.5% of dry matter. All algae contain one or more types of chlorophyll: chlorophyll-*a* is the only chlorophyll in cyanobacteria

(blue-green algae) and rhodophyta. Like all higher plants, chlorophyta and euglenophyta contain chlorophyll-*b* as well; chlorophylls -*c*, -*d* and –*e* can be found in several marine algae and fresh-water diatoms. Also micro algae are rich in carotenoids**,** which have the ability to act as provitamin A at the level 0.1– 0.2% of dry matter (Becker, 1994). The main carotenoids produced by micro algae are β-carotene from *D. salina* which has a content of up to 14% dry matter (Becker, 1994; Spolaore *et al*., 2006a) and astaxanthin from *H. pluvialis* whose content is 1.5–3.0% of dry matter and astaxanthin which has a powerful antioxidant, more effective than vitamins C and E or other carotenoids (Mata *et al*., 2010).

Carotenoids are characterized by the ability to quench singlet oxygen, the inhibition of peroxide formation, and the correlation of antioxidant dependency with oxygen partial pressures. The ketocarotenoids, such as astaxanthin and canthaxanthin, were the best radical scavengers that did not contain conjugated terminal carbonyl functions. These findings suggest that the keto function in conjugation with the polyene backbone is able to stabilize carbon centered radicals more effectively than the polyene backbone alone (Jackson *et al*., 2008). Blas Valdivia (2012) reported that micro algae produce also terpenes in the form of carotenoids. These compounds offer therapeutic effects. Carotenoids are tetraterpenoid organic pigments that are naturally occurring in the chloroplasts and chromoplasts of photosynthetic organisms.

The micro algae have developed defiance system against photooxidative damage by antioxidative mechanisms to detoxify and eliminate these highly reactive oxygen species. These antioxidant defiance system includes hydrophobic (carotenoids & α-tocopherol) and hydrophilic antioxidant (ascorbic acid & glutathione) and antioxidant enzymes likes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) ascorbic peroxidase and peroxidase (Rao *et al.*, 1996; Malanga *et al.*, 1997; Rijstenbil, 2002).

This pigment has many benefits such as anti-inflammatory and immune enhancing properties in humans and animals. Also it is widely used in fish feeds and particularly in salmon feeds (Spolaore *et al*., 2006a; Gouveia *et al*., 2008; Mata *et al*., 2010). Some reports support the assumption that daily ingestion of astaxanthin may protect body tissues from oxidative damage as this might be a practical and beneficial strategy in health management. It

has also been suggested that astaxanthin has a free radical fighting capacity worth 500 times that of vitamin E (Dufossé *et al*., 2005).

2.3.6 Micro algae as a source of antioxidant

The antioxidants are substances that protect cells from free radicals. Several antioxidants have been isolated from micro algae. Hence, there is increasing interest in using micro algae as natural antioxidants source functional food/nutraceuticals. Natrah *et al*. (2007) reported a stronger antioxidant activity exhibited by methanolic micro algae crude extracts (from *e.g. Isochrysis galbana, C. vulgaris, Nannochloropsis oculata, Tetraselmis tetrathele, Chaetoceros calcitrans*) when compared with α-tocopherol, but lower than the synthetic antioxidant butylated hydroxytoluene (BHT). However, synthetic antioxidants as BHT and butylated hydroxyanisole (BHA), are questionable in terms of their safe use, since they are believed to be carcinogenic and tumorogenic if given in high doses (Schilderman *et al*., 1995; Aruoma, 2003). Moreover, the natural food antioxidants have higher bioavailability and, therefore, exert a better protective effect than the synthetic ones (Spolaore *et al*., 2006a; Gouveia *et al*., 2008).

In recent years, it has been demonstrated that micro algae of the Chlorophyceae class could be excellent nutraceuticals because they contain polyphenols, chlorophyll, α-carotene, ascorbic acid, lycopene, α-tocopherol, xanthophylls, and PUFAs. For this reason, some research groups have studied the nutraceutical properties of the genera *Dunalliela*, *Haematococcus*, and *Chlorella* (Blas Valdivia, 2012).

Polyphenols have different nutraceutical properties, such as an antioxidant, antiinflammatory (Biesalski, 2007), anticancer (Oz and Ebersole, 2010), antibacterial (Du *et al*., 2011), antiatherogenic, and antiangiogenic (Rimbach *et al*., 2009). There are now polyphenols with therapeutic properties for which the mechanism of action at the molecular level has been discovered and they are used in clinical trials.

Chlorella contains antioxidants like flavonoids which suppress the formation of reactive oxygen species (ROS) either by inhibiting enzymes or chelating trace elements involved in free radical production. Thus, flavonoids help maintain a ROS steady state in the

case of physical and chemical injury of the cell (Corradini *et al*., 2011). Not all flavonoids are ROS scavengers because some flavonoids, as nucleophiles, trap electrons from the ROS and become a free radical themselves, which then propagate a chain reaction causing a deleterious effect in the cell (Grassi *et al*., 2009). Flavonoids offer cardiovascular protection because of their indirect inhibition of the angiotensin-converting enzyme (ACE; EC 3.4.15.1) (Actis Goretta *et al*., 2006). Other enzymes inhibited by flavonoids are aromatase (EC 1.14.14.1) and α -amylase (EC 3.2.2.1) (Hargrove *et al*., 2011). The inhibition of enzymes that have a Fe-S cluster has been demonstrated (Mena *et al*., 2011).

Many studies have focused on the biological activities of plant derived polyphenolic flavonoids as they are well known to exhibit antioxidant activity through a variety of mechanisms including scavenging of ROS, inhibiting lipid peroxidation as well as chelating metal ions while the phenolic compounds are reported as an index of antioxidant function (Maulik *et al*.,1997). Composition analysis (total antioxidant capacity, total phenols, flavonoids, carotenoids, Chlorophyll and some vitamins of *Chlorella* showed in Table 2.3.6.1 Dietary antioxidants like vitamin C, vitamin E, and β-carotene are among the most widely studied dietary antioxidants. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E, a major lipid-soluble antioxidant, is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane FA's from lipid peroxidation. Vitamin C has been cited as being capable of regenerating vitamin E. β-carotene and other carotenoids are also believed to provide antioxidant protection to lipid-rich tissues. Research suggests that β-carotene may work synergistically with vitamin E. A diet that is excessively low in fat may negatively affect β- carotene and vitamin E absorption, as well as other fat-soluble nutrients.

Table 2.3.6.1 Composition analysis of total antioxidant capacity, total phenols, flavonoids, Chlorophyll**,** carotenoids and some vitamins of *Chlorella*.

Parameters	***Chlorella***
Total antioxidant capacity	192 µmoles of trolox/10g*
Total antioxidant capacity	596 µmoles of trolox/10g**
Total phenols	24.7 mg/ 10g dry weight
Flavonoids	18.6 mg/ 10g
Vitamin A activity	5.55 IU/10g
B-carotene:	18.08 mg/10g
Chlorophyll a	147 mg /10g
Chlorophyll b	61 mg/10g
Thiamine (vitamin B_1)	0.15 mg/10g
Riboflavin (vitamin B_2)	0.48 mg/10g
Pyridoxine (vitamin B_6)	0.17 mg/10g
Vitamin B_{12}	12.6 µg/10g
Vitamin C	1.6 mg/10g
Vitamin E	0.1 IU/10g
Niacin	2.4 mg/10g
Pantothenic acid	0.13 mg/10g
Folic acid	0.27 µg/10g
Biotin	19 µg/10g
Inositol	16.5 mg/10g

Adapted from sources, (Geetha *et al*., 2010 and Goiris *et al*., 2012)
Total antioxidant capacity in **C. pyriondosa,* in ***C. vulgaris*

Recently, endogenous marine peptides have opened new scenario in advance to develop pharmaceutical agents (Kim and Wijesekara, 2010). Therefore, there has been a growing demand to isolate new functional proteins or bioactive peptides from marine micro algae (Harnedy and FitzGerald, 2011). Over the years, biological activities of enzymatic extracts from marine algae were considered significantly. Bioactivities of the proteolytic enzyme

extracts are based on their inherent amino acid compositions and sequences, and it may vary from two to twenty amino acid constituents, accordingly (Meisel and FitzGerald, 2003). Besides, some particular interests have been seen on the marine algae peptides due to its specific health benefits (Harnedy and FitzGerald 2011). Some of research work has focused on antioxidant protein hydrolysates or peptides which are extracted by organic solvents from marine algae. Moreover, certain sequences of amino acids with covalent bonds and associated thermal stabilities have gained effective antioxidant properties (Sheih *et al.*, 2009).

Several studies also confirm that low molecular weight hydrolysates have more potency to possess ROS scavenging activities than high molecular weight hydrolysates (Chang *et al*., 2007). In addition, Sheih *et al.* (2009) showed that potent antioxidative activity of pepsin hydrolysate from *C. vulgaris* protein waste, generated during production of essence. Further, the isolated and purified peptide (VECYGPNRPQF) with a low molecular mass (1309 Da) was exhibited significant tolerance effect against gastrointestinal enzymes. Furthermore, the purified peptide from algae protein has shown higher (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical scavenging (IC50 9.8 ± 0.5 μM) and superoxide radical scavenging (IC50 7.5 ± 0.12 μM) activities by comparing with the standard antioxidants. Antioxidative peptides are associated in 5–11 amino acid residues including proline, histidine, tyrosine or tryptophan and hydrophobic amino acids in milk protein (Pihlanto, 2006). In addition, two antioxidant peptides, carnosine and glutathione, also have been found in macroalgae. However, these peptides are present in high concentrations in animal muscles.

Bioactivities peptides have been described as mimic hormones, or showing drug like activities. They could alter the physiological functions or raise a positive impact through binding to specific receptors and interact on target cells or inhibition by enzyme actions (Fitz Gerald and Murray, 2007; Kitts and Weiler, 2003). Some micro algae species have induced biological activities, which are associated with proteins, protein hydrolysates or peptides, that can affect their standing beyond their nutritional values as antioxidant (Karavita *et al*., 2007; Kim *et al*., 2006), antihypertensive (FitzGerald and Murray, 2007), immune-modulatory (Morris *et al*., 2007), anticancer (Sheih *et al*., 2010).

Recently, bioactive peptides from enzymatic hydrolysis of various food proteins such as soy protein, casein, whey protein, gelatin and wheat gluten have been shown to possess antioxidative activity (Elias *et al*., 2008). However, antioxidative peptides from marine food sources are gaining attention as new antioxidative alternatives in the last few years (Je *et al*., 2005; Mendis *et al*., 2005; Rajapakse *et al*., 2005; Qian *et al*., 2008a,b). Algae protein waste is a by-product derived from water-extraction process of micro algae, *C. vulgaris* during micro algae essence manufacturing. The pepsin hydrolysate from micro algae protein waste exhibited antioxidative activity in preliminary experiments, indicating that micro algae waste might become a new protein source for selection of novel antioxidative peptides.

2.4 Chlorella as a nutritional supplement

Chlorella is a natural whole food supplement derived from a superior species of fresh water single. The name of *Chlorella* derived from the Greek, "chloros" meaning green and "ella" meaning small (Randall and Cynthia, 2001). There are several species of *Chlorella*, but the most commonly found in supplements are *C. pyrenoidosa and C. vulgaris.* It is estimated that over 10 million people worldwide take *Chlorella* supplements every day to maintain overall health. *Chlorella* is important as a health promoting factor on many kinds of disorders such as gastric ulcers, wounds, constipation, anaemia, hypertension, diabetes, infant malnutrition and neurosis (Yamaguchi, 1997). It is also attributed a preventive action against atherosclerosis and hypercholesterolemia by glycolipids and phospholipids, and antitumor actions by glycoproteins, peptides and nucleotides (Yamaguchi, 1997). However, the most important substance in *Chlorella* seems to be a beta 1, 3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore *et al*., 2006b).

Chlorella vulgaris is a genus of single-celled (unicellular) green algae belonging to the phylum Chlorophyta. It is spherical in shape, about 2 to 10 μm in diameter, and without flagella. It is a good source of nutrients such as valuable protein, calories, fat, and vitamins (Belasco, 1997). Under certain growing conditions, *Chlorella* yields oils high in polyunsaturated fats. It also produces astaxanthin, canthaxanthin, and, in minor amounts, β-

carotene (Mendes *et al*., 2003; Gyenis *et al*., 2005). *Chlorella vulgaris* contains other antioxidants such as lutein and chlorophyle. It is a rich nutritional ingredient because it contains 61.6 % proteins, 12.5 % fat, 13.7 % carbohydrates, trace elements (aluminum, selenium, phosphorus, zinc, calcium, and magnesium), and vitamins (thiamine, B1, B2, B6, ascorbic acid, D, E and K) (Valdivia *et al*., 2011).

Chlorella pyrenoidosa, a fresh water unicellular algae rich in macro and micronutrients, has been used as a food source and nutritional supplement for centuries (Kay, 1991). Animal studies have demonstrated that *C. pyrenoidosa* affects the immune system by stimulating an increase in number and activities of macrophages and polymorphonuclear leukocytes (Kojima *et al*., 1973; Miyazawa *et al*., 1988; Tanaka *et al*., 1986; Konishi *et al*., 1985; Komiyama *et al*., 1986). An acidic polysaccharide prepared from *C. pyrenoidosa* cell wall has also been shown to induce the production of interferon *in vitro* and in mice (Umezawa *et al.*, 1982) and therefore, part of *C. pyrenoidosa's* anticancer effect in part may be mediated through the actions of this cytokine.

2.5 Micro algae in animal nutrition

Micro algae can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals. Nowadays, it is estimated that approximately 30 % of algae produced worldwide is used in animal nutrition, mainly due to their high quality of protein (Becker, 2007). Several micro algae (*e.g. Chlorella*, *Tetraselmis*, *Spirulina*, *Nannochloropsis*, *Nitzchia*, *Navicula*, *Chaetoceros*, *Scenedesmus*, *Haematococcus*, *Crypthecodinium*) can be used in both terrestrial and aquatic animal feed (Harel and Clayton, 2004).

Feeds can be formulated by using vegetable protein sources, vegetable oil sources, fishmeal, mineral and vitamin premixes in order to reach appropriate nutritional properties for each animal group and promote health and welfare benefits (Harel and Clayton, 2004). Using even very small amounts of micro algae biomass can positively affect the physiology of animals by improving immune response, resulting in growth promotion, disease resistance, antiviral and antibacterial action, improved gut function, probiotic colonization stimulation,

as well as by improving feed conversion, reproductive performance and weight control (Harel and Clayton, 2004). The external appearance of the animals may also be improved, resulting in healthy skin and a lustrous coat, for both farming animals (poultry, cows, breeding bulls) and pets (cats, dogs, rabbits, ornamental fishes and birds) (Certik and Shimizu, 1999).

Since feed corresponds to the most important exogenous factor influencing animal health and also the major expense in animal production, the use of alternative high quality protein supplements replacing conventional protein sources is encouraged. Considering that animal feed stands at the beginning of the food chain, increasing public and legislative interest is evident, especially considering intensive breeding conditions and the recent trend to avoid "chemicals" like antibiotics (Breithaupt, 2007). The large number of nutritional and toxicological evaluations already conducted has demonstrated the suitability of micro algae biomass as a valuable feed supplement (Becker, 1994).

The effects of algae supplementation have to be valued with respect to two different groups of animals: ruminants and non-ruminants (monogastric vertebrates). Ruminants (cattle, sheep and goats) are capable of digesting cellulosic material, hence offering the at least theoretical possibility to feed native micro algae directly to these animals, which has not gained much attraction so far. For monogastris, including humans, the algae biomass has to be processed properly before it is made suitable as feed.

2.5.1 Effect of micro algae on ruminants

Most of the studies have no analogous to the concentration needed to produce the benefits with micro algae and different amounts were used for example, Moreno- Indias *et al*. (2014) used 5g/day for goats; Bichi *et al*. (2013) used 8 g/kg of dry matter for dairy ewes; Toral *et al*. (2010) used among 8 and 24 g/kg of dry matter for dairy ewes ; Papadopoulos *et al*. (2002) used among 23.5 and 94 g/ration of micro algae for dairy ewes; Christaki *et al*. (2012) used 40 g/cow; and AbuGhazaleh *et al*. (2009) used up to150 g/cow.

One of the studies on the utilization of fresh untreated micro algae for feeding ruminants has been reported from Bulgaria (Ganowski *et al*., 1975): one liter of concentrated native *S. obliquus* ($2–3\times10^8$ cells/ ml) were fed to calves over a period of three weeks. This

feeding increased the contribution of the intestine in the digestive process without facilitating the digestion of the feed. Only minor differences were observed for digestibility between control and experimental animals.

In a study with drum-dried algae, beef steers were used as experimental animals. The rations tested were composed of alfalfa/hay (2:8), algae/hay (2:8) and algae/hay (4:6) (Hintz and Heitmann, 1967). Since the consumption of the ration containing the higher amount of algae was poor, for better comparison the intake of the other diets was restricted to the daily intake of the algae ration. The addition of algae at both levels did not decrease the digestibility of the crude protein (74 %), but the feed with the higher algae content showed a reduction of carbohydrate digestibility from 68 to 52 %, in agreement with the results obtained in similar trials with sheep. In a study that examined the effects of supplementing dietary dry matter with de-fatted *Lithothamnium calcareum* meal in Holstein cows, up to 1 % algae meal in the diet mediated venous acid–base balance after acidosis induction, but did not improve the tract digestibility or growth performance of the cows (Lopex *et al*., 2013).

Chowdhury *et al*. (1995) fed fattening calves a suspension of micro algae (*Chlorella and Scenedesmus* 5–10 ml volume of cells/l water) at approximately 10% of body weight and found improvement in digestibility of crude fiber and reduced total feeding cost, compared with animals fed with sesame seed oil.

Finishing wethers maintained similar growth performance and carcass characteristics (longissimus muscle area, dressing percentage, marbling score, hot carcass weight, and subcutaneous adipose depth) when fed up to 20% defatted algae biomass on a dry matter basis as a protein replacement, in comparison with wethers fed a control diet (Dib MD *et al*., 2012). In lambs, dietary micro algae increased the n-3 FA content in meat and blood, respectively (Cooper *et al*., 2004; Hess *et al*., 2012).

From the investigation on use of micro algae with ruminants, it should be expected that ruminants represent the group of animals most suitable for feeding with micro algae, since these animals are able to digest even unprocessed algae material (*e.g.* cell walls). However, a limited number of trials have been done due the large amount of micro algae required to

perform appropriate feeding experiments with these animal species. Sheep and cattle show an inability to digest efficiently the carbohydrate fraction of the micro algae (*Chlorella*, *Scenedesmus obliquus* and *Scenedesmus quadricauda*) (Hintz *et al*., 1966; Davis *et al*., 1975). Better digestibility was obtained with *Spirulina* constituting 20% of a complete sheep diet. Calves revealed a minor difference between control and untreated fresh *Scenedesmus* alga fed animals (Calderon *et al*., 1976).

2.5.1.1 Effect of micro algae on rumen microflora

Rumen biohydrogenation is the microbial saturation of dietary unsaturated fatty acids (USFA), which limits the availability of health-associated PUFA's in ruminant meat and milk. Therefore, understanding the ruminal biohydrogenation process is important to generate healthier ruminant products. Interest in research on microorganisms involved in rumen biohydrogenation of linoleic (C18:2n-6) or linolenic (C18:3n-3) acid is currently growing. Polan *et al*. (1964) reported that there was associated between rumen biohydrogenation activity and a *Butyrivibrio fibrisolvens* strain. Since then, several researchers have confirmed the active role of *Butyrivibrio* species in the partial or complete biohydrogenation of unsaturated C18:0 fatty acids (Jenkins *et al*., 2008). Bacteria involved in C18:0 biohydrogenation are grouped in the "*Butyrivibrio* group," which includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* and the species *Clostridium proteoclasticum* (Paillard *et al*., 2007). The last was recently proposed for reclassification as *Butyrivibrio proteoclasticus* (Moon *et al*., 2008), and hereafter, we refer to *C. proteoclasticum* as *B. proteoclasticus*. Although isolation and pure-culture studies have provided fundamental insight into the bacteria involved in rumen biohydrogenation, the relative importance of individual strains in the *in vivo* ruminal lipid metabolism remains largely unclear (Palmquist *et al*., 2005). Indeed, complete *in vivo* biohydrogenation is thought to be a synergistic process involving a consortium of bacteria, each having a share in the conversion of unsaturated to more saturated fatty acid (SFA) (Harfoot and Hazewood, 1997).

Marine products, such as fish oil and algae, proved to possess high effectiveness in the inhibition of rumen biohydrogenation of USFA's (Boeckaert *et al*., 2007; Scollan *et al*., 2001). The long-chain PUFA EPA and/or DHA was found to be the active compounds in this

process (AbuGhazaleh and Jenkins, 2004; Boeckaert *et al*., 2007). Their supplementation reduced C18:0 production, resulting in the accumulation of various hydrogenation intermediates, predominantly C18:1 trans-11 (t11) and C18:1 t10. Incomplete biohydrogenation, induced by dietary algae, was found to be associated with the disappearance of some ciliates (Boeckaert *et al*., 2007).

Currently, the mechanism of biohydrogenation and the microorganisms involved are frequently studied and a growing pool of knowledge is obtained in this field. Several rumen bacteria (*Butyrivibrio fibrisolvens*, *B. hungatei*, *Clostridium proteoclasticum*) have been identified to partially or fully hydrogenate C18:0 fatty acids (van de Vossenberg and Joblin, 2003 and Wallace *et al*., 2006). However, the role of protozoa in the ruminal lipid metabolism is not well defined (Boeckaert *et al*., 2007).

Several *in vivo* studies reported depletion of the protozoal population after addition of PUFA (Czerkawski *et al*., 1975; Broudiscou *et al*., 1994). Next to this, PUFA are also known to affect rumen biohydrogenation (Beam *et al*., 2000). Important sources of long chain PUFA, especially EPA and DHA, are marine products such as fish oil and micro algae. Recently, supplementation of DHA-enriched micro algae was shown to inhibit the *in vitro* hydrogenation of C18:2n-6 and C18:3 n-3, resulting in the accumulation of mainly C18:1 trans-11 and, to a lesser extent, CLA cis-9, trans-11 and C18:2 trans-11, cis-15 (Boeckaert *et al*., 2006).

Dietary supplementation of fish oil previously has shown to promote C18:1 trans accumulation in the rumen (Kitessa *et al*., 2001; Wang *et al*., 2005). Also, *in vivo* trial, supplementation of micro algae to dairy cows altered the rumen FA profile in a similar way as was already observed for fish oil addition (AbuGhazaleh *et al*., 2002). The amount of USFA's increased at the expense of the SFA, with the significant increase in n-3 FA's being related to the administration of DHA enriched micro algae.

Major shifts of the rumen FA profile occurred within the C18:0 fatty acids. Algae supplementation resulted in higher concentrations of C18:3n-3 and C18:2n-6, which is most probably due to a partial inhibition of lipolysis, as accumulation of unesterified C18:3n-3 and

C18:2 n-6 is assumed to rarely occur *in vivo* (Beam *et al*., 2000). Additionally, lower concentrations of C18:3 cis-9, trans-11, cis-15 and higher concentrations of C18:2 trans-11, cis-15 and C18:1 trans fatty acids were observed in the ruminal content of algal-supplemented cows. These results are confirmed by Boeckaert *et al*. (2006) during rumen *in vitro* incubations with C18:2 n-6- or C18:3 n-3-rich oil sources and supplemented with DHA enriched micro algae. The strong accumulation of C18:2 trans-11, cis-15 and C18:1 trans-11 indicates that the algae diet affects the second and third hydrogenation step of C18:3 n-3 (C18:2 trans-11, cis-15 to C18:1 trans-11 to C18:0) and the second hydrogenation step of C18:2 n-6 (C18:1 trans-11 to C18:0), which are steps following the hydrogenation of a conjugated intermediate. Besides the significant increase in C18:1 trans-11, algae supplementation induced accumulation of C18:1 trans-10. Accumulation of the latter most often has been associated with high concentrate based rations and/or low rumen pH (Bauman and Griinari, 2003).

The marine products can change the total bacterial community structure (Kim *et al*., 2008) and they have been proved to possess a protective mechanism against the rumen biohydrogenation of USFA's (Harfoot and Hazewood, 1997; Boeckaert *et al*., 2008c). Algae PUFA remain encapsulated inside the cells of microorganisms and could be protected by the cell membrane (Papadopoulos *et al*., 2002). Boeckaert *et al*. (2008c) found that supplementing dairy cows with micro algae inhibited the total biohydrogenation of C18:2n-6 and C18:3n-3, resulting in increased concentrations of biohydrogenation intermediates in rumen, in particular the 18:1-cis and -trans isomers, whereas C18:0 decreased due to a decrease of the capacity of the *butyrivibrio* community to hydrogenate C18:1 trans FAs.

Besides a strong accumulation of 18:1 trans fatty acids, algae supplementation provoked shifts in the bacterial and ciliate community of the rumen. Changes in the bacterial diversity have also been observed after fish oil inclusion by Huws *et al*. (2006). Boeckaert *et al*. (2007) reported that the shifts in the ciliate Denaturing Gradient Gel Electrophoresis (DGGE) profiles suggest a decreased importance of the ciliates *Isotricha prostoma* and *I. intestinalis* and some species of *Epidinium caudatum* in the rumen of micro algae fed cows. Results for *Eudiplodinium maggii* and *Diplodinium dentatum* were contradictory as some

bands of these ciliates faded away while others became more pronounced upon algae addition. The protozoal population is known to have pronounced effects on the bacterial activities in the rumen (Williams and Coleman, 1992); hence, it is unclear whether this altered bacterial community results directly from algae supplementation or rather is related with a modified and decreased ciliate community as suggested by the ciliate DGGE profiles and q-PCR.

2.5.1.2 Effect of micro algae on fatty acid profile in milk

The research on micro algae biomass supplementation in food producing animals has opened a new gateway to improve human health. In recent years, algae were used in the diet of ruminants to enrich their products with valuable PUFA. The effect of supplementing a basal dairy cow diet of alfalfa hay, maize silage, maize grain and soybean meal with rumen-protected (xylose-coated) and unprotected marine micro algae (*Schizochytrium sp*.) at a level of 910 g/day on the milk FA content was investigated by Franklin *et al*. (1999). The results have shown that the addition of micro algae (*Schizochytrium sp.*) in diets of dairy cows resulted in increased levels of DHA and CLA in milk fat, whereas SFA were reduced. Animals offered the protected micro algae consumed 29.6 g DHA/day and secreted 4.9 g DHA into milk daily, a transfer efficiency of almost 17 %, while a transfer efficiency of 8 % was achieved with cows offered the unprotected marine algae (Franklin *et al*., 1999). This indicated that the rumen protection was effective in presenting a greater amount of DHA for uptake at the mammary gland.

In general, feeding marine micro algae affected the FA profile of the milk fat from Brown Swiss and Holstein cows similarly (Franklin *et al*., 1999). Total USFA were greater in milk fat from cows fed protected micro algae (by coating with xylose) compared to cows fed the control diet and tended to be greater in milk fat from cows fed unprotected micro algae. The alteration in the proportions of SFA was mainly a result of lower C18:0 and greater PUFA, specifically DHA and CLA, in milk fat from cows fed micro algae. Concentrations of C14:0 and C16:0 were greater as well (Franklin *et al*., 1999). Scientists involved in a roundtable discussion (Berner, 1993) of the role of milk fat in human diets agreed that SFA intake should be decreased. Some SFA's are considered to be cholesterol-

raising (C12:0, C14:0 and C16:0), whereas most FA's in milk fat are not. It is unknown whether decreasing the proportion of total SFA's while increasing total USFA's will improve the cholesterol status of consumers if proportions of C14:0 and C16:0 are also increased. The decrease in the proportion of short- and medium-chain FA (C4:0 to C14:0) with oil and micro algae supplementation was reported by others (Donovan *et al*., 2000; Boeckaert *et al*., 2008b) who found similar reductions in de novo FA synthesis in cows fed oil and/ or micro algae, which is probably attributed to greater intake of PUFA. The PUFA's, their biohydrogenation products, or both are potent inhibitors of mammary synthesis of short and medium-chain FA through a direct inhibitory effect on acetyl-CoA carboxylase activity (Bauman and Griinari, 2003). Furthermore, according to Papadopoulos *et al*. (2002), the incorporation 0, 23.5, 47 and 94 g of micro algae (*Schizochytrium sp*.) in the feed of milk producing ewes for 6 weeks resulted in increased levels of milk fat and protein and the enrichment of milk in the PUFA's (DHA and EPA). Regarding ewes dairy products, i.e. yogurt and feta cheese, they were enriched in PUFA's compared with controls (Papadopoulos *et al*., 2002). Moreover, the above researchers pointed out that the storage of feta cheese for a period of 5 months did not influence the FA's content.

Glover *et al*. (2012) reported that feeding micro algae and fresh forage decreased total milk fat content, but elevated its DHA concentration. A parallel study was conducted with sheep to determine if micro algae and (or) their co-supplementation with sunflower oil in the diet could enhance the nutrient profile of milk (Toral *et al*., 2010). While the milk yield was unaffected by the dietary treatments, the milk fat content was decreased and the milk DHA concentration was increased as dietary micro algae concentration rose.

There was only one paper used low fat micro algae (5 g/day of *C. pyrenoidosa*) as a probiotic in dairy goats feeding (Moreno- Indias *et al*., 2014). The study reported that, there was no significant effect of *C. pyrenoidosa* addition on milk fat percentage between treated and control group. In addition, fat percentage decreased throughout the experiment in both groups. Regarding the fatty acid profiles, no differences were observed in individual fatty acids between groups. However, some fatty acids showed a tendency in their evolutions, such as C10:0 and C12:0 which were increased, or C16:0 which was decreased. Also, no

significant effects of *C. pyrenoidosa* diet addition were observed in SFA, MUFA and PUFA percentages and atherogenic index (AI). In addition, there was a trend of raising the SFA percentage and AI and decreasing the MUFA and PUFA percentages in both groups from day 10 to day 40 of lactation. Finally, that study reported that, the addition of 5 g/day of *C. pyrenoidosa* is not enough to show any differences on milk quality parameters and the concentration of micro algae added should be revised.

2.5.2 Others

The potential effects of algae cell wall structure on digestibility to humans and non-ruminant animals has been raised by several authors, as reviewed by Becker (2004). Janczyk *et al*. (2007) tested the digestibility of *Chlorella* biomass in rats using three treatments such as spray-dried, spray-dried and electroporated and spray-dried and ultrasonicated. Ultrasonication was found to increase the protein digestibility of *Chlorella* from 53 % (spray-dried) to 63 %.

Other studies have shown that the micro algae could possess an effect on reproduction. Ishibashi (1971) stated that *Chlorella* influence on estrus in rats that could have been caused by estrogenic substances present in the micro algae cells. Another green alga, *Spirulina*, was shown to increase the number of mice pups in litters in compare to mice fed casein (Kapoor and Mehta, 1993). Similar effect was received when pigs were fed diet containing 5 % of *Spirulina maxima*. The effect was only observed in the first reproduction cycle, whereas in the second cycle the sows fed this diet delivered less piglets in their litters (Fevrier and Seve, 1975). Pabst *et al*. (1978) fed mice diet containing 20 % of *Scenedesmus acutus* over 6 generations, and found a decrease in pups number with accompanying increase in the birth weight. Lipstein *et al*., 1980, found no effect of supplementation of layer diets with *Chlorella*.

2.6 Antioxidant enzymes activity

Antioxidants are required to protect the animal's cells from damage due to the presence of free radicals. In normal cells, free radicals and ROS can be effectively eliminated by an enzyme-mediated system such as SOD, peroxidase, Glutathione peroxidase (GSH-Px) and

also with ascorbic acid. When the homeostasis between the prooxidant formation and antioxidant capacity is disrupted, whereby prooxidant formation exceeds antioxidant capacity, oxidative damage will accumulate and will result in patho-physiological events. Accumulating evidence indicates that active oxygen and free radicals would attack key biological molecules such as DNA, protein, and lipid that lead to many degenerative disease conditions (Suja et al., 2004), such as cancer (Leanderson et al., 1997), gastric ulcers (Debashis *et al*., 1997), Alzheimer's, arthritis and ischemic reperfusion (Vajragupta *et al*., 2000). To maintain the prooxidant–antioxidant balance, the removal of free radicals and ROS is probably one of the most effective defenses of a living body against various diseases (Yang *et al*., 2001; Butterfield *et al*., 2002; Fazlul and Li, 2002). Antioxidants present in the diet can delay lipid peroxidation by inhibiting the initiation or propagation phase of oxidizing chain reactions by scavenging free radical (Sherwin, 1990).

Antioxidants play an important role in the human body by reducing oxidative reactions. Especially, endogenous antioxidant enzymes such as SOD, catalase (CAT), GSH-Px and non-enzymatic antioxidants such as vit. C, α-tocopherol and selenium protect internal organs and tissues from oxidative damage by various toxic reactive oxygen and nitrogen species (Ahn *et al*., 2004). However, imbalances between endogenous antioxidants and ROS lead to cause serious health issues and disorders. (Valko *et al*., 2007). Therefore, researchers are continually seeking for a good source with potent antioxidant ability as an alternative for the dietary supplements. In fact, among the marine fauna and flora, marine micro algae have considered as a rich source of natural antioxidants (Ngo *et al*., 2011).

Mammalian cells are protected against free radicals by enzymatic and nonenzymatic antioxidant defenses (Fridovich, 1978; Meneghini, 1988). The primary antioxidant enzymes are SOD, which catalyzes the dismutation of superoxide anions ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2), CAT and GSH-Px which catalyze the degradation of H_2O_2 to H_2O and O_2. Glutathione peroxidase catalyzes the reduction of H_2O_2 to water at the expense of reduced glutathione (Fridovich, 1978).

2.7 Enzyme activity in plasma and milk

Milk contain vital nutrients such as proteins, carbohydrates, lipids, minerals and vitamins, together with bioactive substances including immunoglobulins, peptides, antimicrobial factors, hormones and growth factors (Grosvenor *et al*., 1993; Clare and Swaisgood, 2000). Over 60 indigenous enzymes have been identified so far in the milk of various mammalian species (Fox, 2003). Mostly on bovine and human milk, showing that indigenous milk enzymes play a key role in regulating lactogenesis, including inducing active involution, and that they are essential components of antioxidation and the innate immune system of milk.

Oxidation of milk causes the development of off-flavours and is a major problem both in fresh milk and in processed dairy products (Nielsen *et al*., 2001). The oxidative stability of milk is a balance between pro- and anti-oxidative factors (Lindmark-Mansson and Akesson, 2000); hence, identification and characterization of these factors are necessary to predict the rate of oxidation.

Milk contains antioxidative enzymes, such as CAT, SOD, and GSH-Px that can reduce oxidative deterioration, but the importance and relative contributions of these enzymes to the oxidative stability of milk is unclear (Lindmark Ma˚ nsson, 2000; Lindmark Mansson and Akesson, 2000). Two distinct classes of GSH-Px are known: Selenium-dependent (EC 1.11.1.9) and Se-independent (EC 2.5.1.18). Both utilise glutathione for reducing organic hydroperoxides, but the former has a selenocysteine residue at the active site whereas the latter utilizes a catalytic tyrosine (Gebicki *et al*., 2002).

Antioxidant enzymes are considered to be the primary defense that prevents biological macromolecules from oxidative damage. Among the antioxidant system, GSH (L-c-glutamyl-cysteinyl-glycine) has an important role as it is a ubiquitous non-protein thiol present in the cell in its reduced form acts as an intracellular reductant. SOD catalyzes the transformation of superoxide radicals to H_2O_2 and O_2, CAT is responsible for the reduction of hydrogen peroxide, and GSH-Px catalyzes the reduction of both hydrogen and lipid peroxides. Thus, by

using GSH as a cofactor, the antioxidant system scavenges the oxygen radicals and protects the biological molecules from damage (Vuchetich *et al*., 1996; Vijayavel *et al*., 2006).

Clinical chemistry laboratories are increasingly measure plasma and tissue biomarkers of oxidative stress and activity of the antioxidant system. There are fully automated assays for SOD, GSH-Px, GR, glucose-6-phosphate dehydrogenase and total antioxidant status (Miller *et al*., 1993). Development of a fully automated CAT assay may be an important addition to a panel of parameters to assess the activity of the antioxidant system.

2.7.1 Catalase (CAT)

Catalase is an extensively studied enzyme produced by a wide spectrum of eukaryotic and prokaryotic organisms (Switala and Loewen, 2002). It is a primary component of the antioxidant system that defends the oxidative stress which is ubiquitously associated with pathologic conditions including cancer, diabetes, cataract, atherosclerosis, ischemic/reperfusion injury, arthritis, neurogenerative diseases, nutritional deficiency and aging. Catalase decomposes H_2O_2 into a major reactive species that in the presence of iron or other metal ions oxidizes cellular biochemicals to cause cytotoxicity (Mates and Sanchez-Jimenez, 1999; Vendemiale *et al*., 1999).

Sun and Oberley (1989) demonstrated inhibition of CAT by GSH and dichlorodiphenyltrichloroethane (DTT), but little inhibition by their oxidized forms. Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) reduced the inhibition caused by these compounds. The inhibition of CAT by GSH was shown to be largely reversible. Yun *et al*. (1995) reported that *C. pyrenoidosa* contain chlorophyllin which inhibits NADPH- cytochrome p450 reductase in the electron transport system cytochrome p450.

Catalase activity may be determined by quantifying the evolution of O_2 manometrically or by titrametrically measuring the reduction of H_2O_2. Catalases are haemcontaining enzymes that are distributed widely in plant, microbial and animal tissues and secretions; liver, erythrocytes and kidney are particularly rich sources. A catalase was among the first enzymes demonstrated in milk. Babcock and Russell (1897) reported that an extract of separator slime

(somatic cells and other debris) could decompose H_2O_2. Moro (1902) did not include catalase among the indigenous enzymes in milk, but Grimmer (1926); Corbin and Whittier (1965) included references from 1901 and 1907, respectively, which indicated the presence of catalase in milk. According to Shahani *et al*. (1973), Fox and Morrissey (1981), catalase was first reported in 1907, but according to Whitney (1958) the presence of catalase in milk was not reported until 1911. The reason for this uncertainty is unclear since catalase is easily detected; perhaps, the problem is one of nomenclature, as many early workers used general terms such as "oxidase" and "reductases".

According the results of Daniel Boismenu *et al*. (1989) the CAT activities range from 9.3 to 264.4 U/ml. This higher value in the variation coefficient reflects the limitation of the spectrophotometric method for solutions of low activity. This is caused by the fact that the diminution of absorbency becomes smaller as the concentration is decreased.

2.7.2 Lactoperoxidase (LPO)

Milk contains a variety of compounds that protect the neonate as well as the milk itself from a host of deleterious microorganisms (van Hooijdonk *et al*., 2000; Clare *et al*., 2003; Floris *et al*., 2003). One of those compounds is the enzyme LPO that is able to form oxidized halides and pseudohalides, which are potent biocidal small molecules. Although its name distinctly points to a dairy origin, LPO is also present and active in many more secretory fluids in various parts of the body. The earliest reference to LPO dates back to 1924 (Hanssen, 1924) and ever since the enzyme has been extensively studied and described in the scientific literature.

Lactoperoxidase is one of the most abundant milk enzymes in natural form, and it represents approximately 1 % of the proteins in whey (Reiter, 1985). Its activity can be affected by many factors, such as animal species (Pruit and Reiter, 1985), breed and lactation stage (Zapico *et al*., 1991), in addition to the individual differences (Medina *et al*., 1989).

Lactoperoxidase is a milk protein with antimicrobial function. It is an innate immune factor, which is synthesized and released from alveolar epithelial cells (Harada *et al*., 1973) and neutrophils (Moldoveanu *et al*., 1982). Lactoperoxidase activity in milk was up regulated

by lactoperoxidase system inoculation into the mammary gland of cows (Isobe *et al*., 2009). Peroxidase in rat tracheal glands was reported to be increased as a result of exposing the rats to microorganisms (Kinbara *et al*., 1992). In contrast, LPO appears to be constitutively present in milk (Marshall *et al*., 1986; Fonteh *et al*., 2002; Isobe *et al*., 2009). However, little is known about factors regulating its expression and activity (Fragoso *et al*., 2009).

Fonteh *et al*. (2002) reported that LPO activity exhibited a cyclic pattern with alternating peaks and troughs throughout lactation and that extremely large variations were observed between and within cows. However, no precise investigation has been done on the possibility of milk LPO as an indicator of mastitis. Cooray (1994) reported bovine myeloperoxidase as an indicator of mastitis in dairy cows.

Previous reports have indicated different levels of LPO activity in goat milk. Zapico *et al*. (1990) reported LPO activity of 1.55 U/ml in raw goat milk from Spain. The LPO activity of Creole goat milk were found to be 4.45 U/ml (Saad de Schoos *et al*., 1999). According to Marshall *et al*. (1986) LPO activity was increased from 1.44 U/ml at 14 d before drying-off to 3.28 U/ml at 7 d after drying-off, but decreased to 0.87 U/ml within 18 d after kidding.

2.7.3 Glutathione S-transferase (GST)

Glutathione *S*-transferase has been shown to catalyze the transformation of a wide variety of electrophilic compounds to less toxic substances by conjugating them to glutathione and also function as scavenger proteins for many reactive intermediates (Habig *et al*., 1974; Hunaiti *et al*., 1995b). Glutathione S-transferase consists of a family of enzymes that contributes to the cellular detoxfication by catalyzing GSH conjugation to electrophilic substrates, including carbonyl compounds like 4-hydrosyalkenals, which are toxic products of lipid peroxidation (Danielson *et al*., 1987; Mannervik and Danielson, 1988).

Oliveira *et al*. (2009) reported that some species of micro algae contain heavy metals at relatively high quantities such as cadmium, chromium, nickel and vanadium. Reddy *et al*. (1981) reported that heavy metals inhibit the enzyme by association with the sulfhydryl group of a sensitive cysteine. Moreover, a decrease in GST activity has been reported in lead intoxication (Meyer *et al*., 1991), and in the blood of lead-exposed workers (Hunaiti *et al*.,

1995a). The results of several studies have indicated that oxidative damage to hemoglobin may be induced by lead and lead-induced hemolysis is associated with peroxidation of erythrocyte membranes (Clemens and Waller, 1987; Sugawara *et al*., 1991; Hunaiti *et al*., 1995a). Hunaiti and Soud (2000) showed that negative correlations were observed between lead concentrations and the contents and regeneration rate of glutathione as well as the activities of GST, GSH-Px and GR.

2.7.4 Glutathione peroxidase (GSH-Px)

This is one of the most important enzymes in the body with antioxidant properties. Levels of GSH-Px in the body are closely linked with that of glutathione, the master antioxidant. Glutathione is a tripeptide that not only protects the cells against ill effects of pollution; it also acts as body's immune system booster. It is present in high concentrations in the cells and plays a pivotal role in maintaining them in reduced state lest they suffer damage by oxidation from free radicals. The role as antioxidant is particularly important for brain as it is very sensitive to presence of free radicals. Combination of certain antioxidants like glutathione, vitamin C and E, selenium and glutathione peroxidase are very powerful in helping the body fight against the free radicals (Praveen and Ashish, 2012).

There are several reports of GSH-Px activity in milk, but only indirect evidence using antisera specific for extracellular GSH-Px points to the existence of this form in bovine milk. The only direct evidence for the presence of GSH-Px in milk is purification of the enzyme from human milk (Bhattacharya *et al*., 1988). The existence of multiple GSH-Px was not recognized at that time and, unfortunately, classification of the purified enzyme based on the reported amino acid composition is ambiguous. Thus, neither the established sequences of human extracellular GSH-Px (Takahashi *et al*., 1990) nor intracellular GSH-Px (Sukenaga *et al*., 1987) match the amino acid composition reported for the purified GSH-Px from human milk obtained by (Bhattacharya *et al*., 1988).

With GSH being oxidised by sulphydryl oxidase, which is far more active in milk than GSH-Px, alternative reducing substrates must be used if GSH-Px is to be of any significance as an antioxidative enzyme in milk. Similar arguments have been put forward for the

significance of extracellular GSH-Px in plasma, in which the concentration of GSH also is in the low micro molar range (Esworthy *et al*., 1993). Both thioredoxin and glutaredoxin have been shown to be efficient electron donors for extracellular GSH-Px (Bjornstedt *et al*., 1994; Esworthy *et al*., 1993), and if either of these small proteins are present in milk, and if thioredoxin reductase and sufficient NADPH are also available, this system could provide GSH-Px with substrate to act as an antioxidant. However, data with reduced activity of intracellular GSH-Px added to milk further indicate that the conditions in milk are unfavorable for GSH-Px activity.

Wullepit *et al*. (2011) reported that, the plasma α-tocopherol concentration remained unchanged when micro algae were added to the periparturient diet, whereas ferric reducing ability of plasma and GSH-Px levels indicated a numerical improvement of the oxidative status. On the other hand, the ALG group showed a significantly higher mean thiobarbituric acid reactive substances level, which was most distinct at parturition. This means that micro algae supplementation tends to increase lipid peroxidation, and this is especially the case when cows are more susceptible to oxidative stress. It has been reported earlier that high dietary levels of PUFA are a risk for increased susceptibility to oxidative stress, especially when no extra antioxidants are supplemented (Halliwel and Chirico, 1993; Sarkadi Nagy *et al*., 2003; Gobert *et al*., 2009). Other studies (Kesavulu *et al*., 2002; Barbosa *et al*., 2003) hypothesized a possible relationship between n-3 FA's and the reduction of oxidative stress. They reported that n-3 FA's (like DHA) might protect tissues from oxygen damage, exert antioxidant enzyme activities and even help to prevent lipid peroxidation. However, these results were obtained from studies with rats and human. It could be that these effects are different in dairy cows, e.g. due to ruminal biohydrogenation (Kesavulu *et al*., 2002; Barbosa *et al*., 2003). Wullepit *et al*. (2011) found no clear indication that the micro algae supplementation could improve the oxidative status of the cows or diminish the susceptibility to oxidative stress around parturition. Otherwise, Wullepit *et al*. (2011) indicated that periparturient dairy cows supplemented with micro algae experienced increased global lipid peroxidation, admittedly leaving the other oxidative status parameters roughly unchanged.

2.7.5 Superoxide dismutase (SOD)

The antioxidant enzyme SOD provides an important means of cellular defense against free radical damage. Extracellular SOD is a secretory glycoprotein (Marklund, 1982) which is synthesized by several cell types, e.g. fibroblasts, endothelial cells, epithelial cells, alveolar type II cells, macrophages, glial cells and smooth muscle cells (Marklund, 1990; Luoma *et al*., 1998). The enzyme catalyses the dismutation of O^{-}_{2} to H_2O_2 and therefore reduces the amount of free radicals in the extracellular space. It has been suggested that SOD may be important for the oxidative stability of milk (Hill, 1975; Asada, 1976; Holbrook and Hicks, 1978; Korycka-Dahl *et al*., 1979; Kankare and Antila, 1982).

In milk, the superoxide anion can be generated from several sources, among them being xanthine oxidase (Pederson and Aust, 1973; Hodgson and Fridovich, 1976) and lactoperoxidase (Johnson, 1974; Hill, 1977). The superoxide anion reacts slowly with hydrogen peroxide (Ferradini *et a1*., 1977; Koppenol *et a1*., 1977) but the reaction is accelerated by ferric ions (Czapski and Iian 1977; Koppenol *et al.*, 1977). Dismutation of the superoxide anion by SOD and deactivation of hydrogen peroxide by CAT may therefore be of importance in preventing lipid oxidation.

Several authors have suggested that SOD may act as an antioxidant in milk under normal storage conditions in the absence of light (Holbrook and Hicks 1978; Korycka-Dahl *et a1*., 1979; Hicks, 1980). It is, however, doubtful that the effect of SOD alone is sufficient to explain differences often reported in the oxidative stability of milk. Taking dietary supplements that provide an adequate supply of SOD will be helpful in maintaining overall well being and health because it protects our entire body from the harmful effects of free radicals.

Robert *et al*. (1990) reported that the level of SOD activity in blood plasma in human and rats were about 33.3 and 26.3 U/ml respectively. Laukkanen *et al*. (2000) reported that the level of SOD activity in rabbit blood was 17 U/ml. To my knowledge there was no values in ruminant about the level of SOD activity in blood plasma.

2.7.6 Glutathione reductase (GR)

Glutathione reductase catalyzes the reduction of glutathione disulfide to glutathione, a major cellular antioxidant. It is essential for host defense against the gram- negative bacteria *Escherichia coli* in a mouse model of sepsis (Yan-Jing *et al*., 2013). Glutathione, an important water-soluble antioxidant, is synthesized from the amino acids glycine, glutamate, and cysteine. Glutathione directly quenches ROS such as lipid peroxides, and also plays a major role in xenobiotic metabolism. The antioxidant defense system in micro algae includes besides carotenoids, α-tocopherol and ascorbic acid the glutathione (Rao *et al.,* 1996; Malanga *et al.,* 1997; Rijstenbil, 2002).

2.7.7 Plasma protein

High albumin levels have been shown to profoundly extend the life span of cells *in vitro* and to prevent the mutation of cancer lines. This finding is quite relevant considering that albumin is part of the antioxidant pool it being a free radical scavenger (Halliwell, 1988). *Chlorella pyrenoidosa* is the only known natural substance to raise albumin levels. On the other hand, optimal protein levels indicate sound kidney and liver function and an unstressed immune system while low albumin levels are proving to be an extremely accurate predictor of morbidity and mortality.

Su-Ching *et al*. (2012) found that in rats the group 1 and group 2 fed diet containing 4 and 8 % *Chlorella* had higher albumin concentration than the control group which had no *Chlorella*. The *Chlorella* polysaccharides increase the production of nitric oxide and nitrogen dioxide in macrophages enhancing the innate immune response, mediated by Toll-like receptors (TLR-4) (Suarez *et al*., 2010).

Serum protein, especially albumin, is extremely accurate indicator of overall health status, and that low albumin levels exist at the onset and progression of virtually every non-hereditary degenerative disease process, including cancers and cardiovascular heart disease.

2.8 Microbial enzyme activity in rumen

Unlike non-ruminant animals, ruminants have an extensive array of microbial enzymes produced in the rumen, and these enzymes play an important role in the ruminant digestive process. The most extensively studied enzyme systems of the rumen are those involved with the digestion of fiber and other associated or related plant cell wall polymers. Fibrolytic activity in the rumen arises from protozoal, fungal and bacterial sources, primarily *Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus flavefaciens* (Forsberg and Cheng, 1992). Ruminal fungi are noted for their production of potent fibrolytic enzymes and their ability to degrade even the most recalcitrant plant cell wall polymers (Forsberg and Cheng, 1992 and Forsberg *et al*., 1993).

The enzyme activities confirmed to exist in the rumen are diverse, and include plant cell wall polymerdegrading enzymes (e.g. cellulases, xylanases, â-glucanases, and pectinases), amylases, proteases, phytases and specific plant toxin-degrading enzymes (e.g., tannases). The variety of enzymes present in the rumen arises not only from the diversity of the microbial community but also from the multiplicity of fibrolytic enzymes produced by individual microorganisms (Doerner and White, 1990 and Yanke *et al*., 1995).

Efficient digestion of complex substrates in the rumen requires the coordinated activities of many enzymes. Two models have been proposed for individual cells to describe the organization of fibrolytic enzyme systems following synthesis and secretion from the cell. In the first model, enzymes act individually and synergistically to effect the hydrolysis of cellulose. (B´eguin and Aubert, 1994 and Wood, 1992). In the second model, the individual enzymes are assembled into multi-enzyme complexes (e.g., cellulosomes). The cellulosomal multi-enzyme complex of the thermophilic bacterium *Clostridium thermocellum* is the most extensively studied example of this model (Bayer *et al*., 1994). High molecular mass complexes containing numerous cellulases have been identified in a number of rumen bacteria, including *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Fibrobacter succinogenes*, and fungi, including *Neocallimastix frontalis* and *Piromyces sp.* (Forsberg *et al*., 1993; Ali *et al*., 1995; Bayer *et al*., 1994; and Fanutti *et al*., 1995).

Algae and fish oil, proved to possess high effectiveness in the inhibition of rumen biohydrogenation of USFA's (Boeckaert *et al*., 2007 and Scollan *et al*., 2001) if they contain long-chain PUFA (EPA) and/or (DHA) because these compounds are active in this process (AbuGhazaleh and Jenkins, 2004; Boeckaert *et al*., 2007). Their supplementation reduce C18:0 production, resulting in the accumulation of various hydrogenation intermediates, predominantly C18:1 trans-11 and C18:1 trans-10. Incomplete biohydrogenation, induced by dietary micro algae, was found to be associated with the disappearance of some ciliates (Boeckaert *et al*., 2007). Most of studies mainly found an inhibition in rumen biohydrogenation and microorganism community by incorporation micro algae in the diet (Boeckaert *et al*., 2007 and Scollan *et al*., 2001). The effect of marine micro algae on microbial population is due to the USFA composition. Some families of micro algae (*Schizochytrium sp.*) are rich in DHA (25 %) and C22:5 (n-6) (12 %), but poor in EPA (1 %) (Barclay *et al*., 1998) while other families (*Chlorella*) are low in DHA (0.22-0.30 %) and EPA (0.30-0.40 %) (Ötleô & Pire, 2001). So, the effect of *Chlorella*, which are low in DHA and EPA, on rumen biohydrogenation and microbial community may be different.

Finally, from this review of literature most of the studies in ruminants has been used micro algae that hight fat content as a source of PUFA's to modify the FA's in milk (Franklin *et al.*, 1999; Papadopoulos *et al.*, 2002; Christaki *et al*., 2012). There was only one paper used low fat micro algae (*Chlorella*) in dairy goats (Moreno- Indias *et al*., 2014) and reported that, the addition of 5 g/day of *C. pyrenoidosa* is not enough to show any differences on milk quality parameters and the concentration of micro algae added should be revised. So our study comes to investigate two strains of *Chlorella* (*C. pyrenoidosa* and *C. vulgaris*) with (10 g/ kg concentrate) as a source of a natural antioxidant in dairy goats to investigate their effect on milk yield, milk composition, enzymes activities and FA profile of blood plasma and milk and on rumen enzymes and microbes.

Part B:
Experiments

Experiment1

3 Experiments

From the literature review, to our knowledge, the last paper used *Chlorella* in dairy goats is carried out by Moreno- Indias *et al*. (2014) who reported that, the addition of 5 g/day of *C. pyrenoidosa* is not enough to show any differences on milk quality parameters and the concentration of micro algae added should be revised. So this experiment comes to investigate the *C. pyrenoidosa* with (10 g/ kg concentrate) as a source of a natural antioxidant in dairy goats on milk yield, milk composition, enzymes activities and FA profile of rumen, blood plasma and milk.

3.1 First experiment: Chlorella pyrenoidosa as a dietary supplement in dairy goats.

3.1.1 Effects of dietary *Chlorella pyrenoidosa* supplementation on enzymes activities and FA profile in rumen, blood plasma and milk of dairy goats

Abstract

Sixteen dairy goats were allocated into two groups (CON and ALG) of eight goats each for 4 weeks to examine the effects of dietary micro algae *C. pyrenoidosa* on milk chemical composition, enzymes activities and FA profile in rumen, blood plasma and milk. The CON group served as control and received a diet without *C. pyrenoidosa*, whereas in the ALG group, 10 g of *C. pyrenoidosa* /kg were incorporated into concentrates. Milk samples were collected weekly for chemical composition. Blood and milk samples were also taken every two weeks to determine the activities of CAT, LPO, GST, GSH-Px, GR and SOD enzymes and FA composition. On the last day of the experiment, rumen samples were collected by a stomach tube 3 hrs after morning feeding for rumen fatty acids analysis. The addition of *C. pyrenoidosa* in ALG did not affect milk chemical composition. In rumen, an increase of accumulation of C18:0 concentration and a decrease in the C18 intermediates, ($P<0.05$) MUFA concentration and ($P<0.05$) C14:1/C14:0 ratio were observed in ALG group. In blood plasma the GR and SOD activities were significantly higher ($p<0.05$ and $p<0.01$

respectively) (0.051 and 17.66 vs. 0.046 and 8.01 U/ml respectively) in ALG compared with those of the CON group. In addition, SOD was shown significantly effects among sampling time ($P<0.01$). Likewise, SOD showed significant interaction between diets and sampling time ($P<0.001$) during the experiment. The plasma protein concentration was significantly higher ($P<0.01$) (10.47 vs. 7.63 mg/ml) in ALG compared to CON. As units per mg of protein: the GR activity was significantly lower ($P< 0.05$) (0.005 vs. 0.006 U/mg protein) in ALG compared with that of CON. In milk, the GSH-Px activity was significantly lower ($P< 0.05$) (0.245 vs. 0.308 U/ml) in ALG compared with those of CON group. In milk of the ALG group, there were significantly decreased some of SCFA (C6:0, C8:0, and C10:0) concentrations and MCFA (C12:0 and C15:0) concentrations and LCFA (C18:0 and C20:0) concentrations compared with those of the CON group while the C4:0 , C14:0 and C16:0 concentrations trend to increase without significant difference between the two groups. In addition, in milk of the ALG group significantly decreased in the concentration of C16:1 ($P<0.05$). Also, in ALG significantly increased in C18:1 trans was observed. It is concluded that, the amounts of 10 g *C. pyrenoidosa* in the diet plays a major role as a natural antioxidant and can increase the levels of protein and the activity of antioxidant enzymes in the animal plasma which may be protect the animal from the harmful effects of free radicals, while the efficiency of transfer the effect of antioxidant to milk enzymes was low. It's also can decrease the proportion of *de novo* FA synthesis due to inhibitory effect of linoleic acid or its metabolites and have not be successful to enrich milk with beneficial FA such as CLA, PUFA and MUFA concentrations may be due to the low content of DHA and EPA in *Chlorella*.

Key words: *Chlorella pyrenoidosa*, plasma, milk, antioxidant enzymes, fatty acid profile, goats

3.1.1.1 Introduction

Milk production from sheep and goats is of great importance for the economy of Mediterranean countries. So, the improvement in dairy sheep and goats farming could have proven to be a suitable alternative for the dairy cattle industry. Oxidation of milk causes the development of off-flavors and causes a major problem in fresh milk and in processed dairy products (Nielsen *et al*., 2001). The oxidative stability of milk is a balance between pro- and anti- oxidative factors (Lindmark-Mansson and Akesson, 2000); hence, identification and characterization of these factors are necessary to predict the rate of oxidation.

To avoid oxidative damage, the body is equipped with a scavenging system such as the GSH-Px (Flohe *et al*., 1976) and SOD (Marklund, 1984). It is important to know that the activity of these enzymes is influenced by nutrition (Aurousseau, 2002). One strategy for producing such foods is alteration of animal diets, using bioactive feed supplements such as micro algae (Christaki *et al.*, 2011; Hoa *et al*., 2011). In fact, micro algae have considered as a rich source of natural antioxidants (Ngo *et al.*, 2011). Micro algae have developed several efficient protective systems against ROS and free radicals (Pulz and Gross, 2004). Natrah *et al*. (2007) reported a stronger antioxidant activity exhibited by *Chlorella* when compared with α-tocopherol, but lower than the synthetic antioxidant butylated hydroxytoluene (BHT).

Chlorella, a type of unicellular green algae, has been a popular foodstuff worldwide. It contains essential amino acids, protein, minerals, vitamins, chlorophyll, and bioactive substances (Borowitzka, 1988). Antioxidant activity is considered to play an important role in the protective effects of *Chlorella*. Endogenous antioxidant enzymes such as SOD, CAT, GSH-Px and non-enzymatic antioxidants such as vit. C, α-tocopherol and Se protect tissues from oxidative damage by various toxic reactive oxygen and nitrogen species (Ahn *et al.*, 2004). However, imbalances between endogenous antioxidants and ROS can cause serious health problems (Valko *et al.*, 2007). The most important substance in *Chlorella* seems to be the β-1,3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore *et al.*, 2006b). Also, *Chlorella* contains carotenoids that characterized by the ability to quench singlet oxygen, inhibition of peroxide formation, and correlation of antioxidant dependency with oxygen partial pressures.

On other hand, adding micro algae can induce further increases in milk CLA content (Palmquist and Griinari, 2006), but also milk fat depression in cows (Franklin *et al.*, 1999; Boeckaert *et al.*, 2008b; Gama *et al.*, 2008). The effects of micro algae are inconsistent and may depend on basal diet composition and algae dosage (Papadopoulos *et al.*, 2002; Reynolds *et al.*, 2006). The micro algae to the livestock feeding are improving the quality of the products enhancing their nutritional value. Changes in the composition of milk and meat from supplemented animals with micro algae have been reported (Moreno-Indias *et al*., 2012).

In human and animal nutrition micro algae have been used; for example in human (Jose and Arley, 2013), poultry (Carrillo *et al.*, 2008), sheep (Travniček *et al.*, 2008), fattening calves (Chowdhury *et al.*, 1995), dairy cows (Christaki *et al*., 2012), and dairy ewes (Papadopoulos *et al.*, 2002). Nevertheless, most of the literature in ruminants has been used micro algae as a source of PUFA's to modify the FA's in milk (Franklin *et al.*, 1999; Papadopoulos *et al.*, 2002; Christaki *et al*., 2012). However, to our knowledge, the effects of low fat micro algae, which are rich in natural antioxidants, has not been studied so far, especially in goats. Thus, the aim of this study was to investigate the effects of supplementation of dairy goats ration with micro algae of the genus *C. pyrenoidosa* on milk yield, milk composition, enzymes activities and FA profile in rumen, blood plasma and milk.

3.1.1.2 Materials and Methods

Animals

The present study was carried out at the experimental farm of Nutritional Physiology and Feeding Department, Faculty of Animal Science and Aquaculture of the Agricultural University of Athens. Sixteen dairy crossbred Alpine goats were allocated into two groups (CON= control and ALG= treated) of eight goats each for 4 weeks. The goats in both groups had similar daily milk yield (CON 1598 g; ALG 1581 g as fat corrected milk 4 %) and average body weight (CON 48.2 kg; ALG 46.6 kg).

Diets

Goat's feeding was based on wheat straw and alfalfa hay as roughage source, and concentrate mixture according to their requirements. Goats in CON served as controls and

received a diet without micro algae, whereas in ALG (treated group) 10g of *C. pyrenoidosa* /kg were incorporated in the concentrates. All goats were fed individually, twice a day, in two equal meals immediately after milking, while they had free access to water. No refusals were left after each feeding. The chemical composition of wheat straw, alfalfa hay, concentrate diet, and *C. pyrenoidosa* and their minerals and FA content is shown in Table (3.1.1.1). Wheat straw, alfalfa hay, concentrate mixture and *C. pyrenoidosa* samples were analyzed for dry matter (DM), ash, crude protein (CP) and ether extracts (EE) according to the Weende procedure (Association of Official Analytical Chemists International, 1990) and for neutral detergent fiber (NDF) and acid detergent fiber (ADF) according to Goering and Van Soest (1970). Trace elements (Se, Sb, Co, Cu, Fe, Pb, Mg, Mn, Mo, Ni, As and Zn) were determined in samples using inductively coupled plasma mass spectrometry, ICP-MS (Perkin Elmer, Elan 9000, Perkin Elmer Life and Analytical Sciences Inc, Waltham, MA, USA). Samples digestion and analytical procedure described by Al-Waeli *et al*. (2012). The total feed, minerals and FA intake of both groups are presented in Table (3.1.1.2).

Milk and plasma samples analysis

Individual milk samples were taken from the goats at 0, 7, 14, 21 and 28 days during the experimental period for chemical analysis. All milk samples were taken at the same day at each sampling time, after mixing the yield from evening and morning milk on a percent volume (5 %). All the milk samples were analyzed for fat, protein and lactose by milkoscan (133/; Foss Electric, Hllerod, Demark).

Milk and blood samples were also collected at 0, 14 and 28 days of the experiment to determine the activities of enzymes and FA analysis. Thirty (30) ml milk sample were collected from each goat, defatted by centrifugation at 13000xg for 4 min and skim milk beneath the cream was gently poured into a clean container. This skim milk is referred to as milk. Freshly prepared milk samples were normally used; otherwise milk was frozen at - 80 ºC. Another thirty (30) ml of milk sample was collected from each goat and frozen at - 80 ºC for FA analysis. At the same sampling days, blood samples of 10 ml were collected from the jugular vein of each goat before the morning feeding in a tube containing potassium ethylene diamine tetra-acetic acid (K-EDTA). Blood samples were immediately centrifuged at 3000

r.p.m for 15 min and the plasma was separated and stored at –80°C until enzymes and FA analysis. On the last day of the experiment, rumen samples were collected by a stomach tube 3 hrs after morning feeding and frozen at -80 ^{0}C for rumen fatty acids analysis.

Table 3.1.1.1 Chemical composition (in g/Kg DM) of wheat straw, alfalfa hay, concentrate mixture, C. *pyrenoidosa* and minerals (in mg/kg DM) and fatty acid profile (g/100 g FA).

Items	**Wheat Straw**	**Alfalfa hay**	**Concentrate mixture**		***C. pyrenoidosa***
			CON	**ALG**	
Chemical composition, g/Kg DM					
DM	934.7	914.7	904.5	904.5	925.4
OM	825.2	822.7	798.8	798.8	869.0
CP	34.3	192.3	123.3	123.3	574.5
EE	14.2	23.0	68.5	68.5	10.3
NDF	791.5	417.2	294.0	294.0	146.8
ADF	448.9	272.6	79.4	79.4	197.8
Minerals, mg/kg DM					
Se	0.073	0.170	0.504	0.522	0.082
Zn	4.57	97.10	121.75	125.00	17.29
Cu	5.06	10.02	16.53	16.58	4.61
Fe	109.32	796.49	394.96	454.09	1711
Mg	1188.03	3014.83	3085.74	2984.60	3494
Mn	9.63	26.18	108.38	121.51	90.10
Mo	0.989	2.776	0.882	0.893	0.371
As	0.101	0.138	0.071	0.113	1.25
Co	0.360	0.486	0.790	0.819	1.399
Ni	0.482	3.538	1.893	2.174	0.332
Sb	0.168	0.093	0.177	0.176	0.601
Pb	0.238	0.277	0.415	ND	0.111
Fatty acid composition (g/100 g FA**).**					
C14:0	6.68	2.88	0.18	0.21	0.71
C16:0	42.23	35.50	18.93	18.31	20.88
C16:1	0.00	2.03	0.19	0.28	6.07
C17:0	3.30	0.55	0.45	0.35	0.52
C18:0	4.03	7.11	4.77	4.72	2.74
C18:1 trans	0.00	0.00	0.56	0.49	0.29
C18:1 cis-9	9.38	0.99	41.35	39.55	4.65
C18:2 trans	0.00	0.00	0.13	0.31	0.14
C18:2 cis	21.46	32.75	30.84	32.97	29.72
C18:3n-6	3.93	0.00	0.00	0.00	0.14
C18:3n-3	0.00	1.15	0.77	0.73	30.09
C20:5n-3(EPA)	8.99	17.04	0.81	1.19	0.21
C20:2	0.00	0.00	0.49	0.41	0.00
C22:2	0.00	0.00	0.00	0.00	1.45
C22:6n-3(DHA)	0.00	0.00	0.00	0.00	0.00
C24:0	0.00	0.00	0.52	0.48	0.20

Table 3.1.1.2 Dry matter, nutrients, minerals, and FA intake by both groups (CON and ALG)

Items	**Group**	
	CON	**ALG**
Total daily intake (g /head/day**)**		
DM	2013	2003
CP	272	271
EE	92	91
NDF	990	978
ADF	499	493
Minerals daily intake (mg /animal/day)		
Se	1.3	1.4
Zn	399.6	401.9
Cu	56.5	56.1
Fe	2326	2411
Mg	13035	12745
Mn	258	279
Mo	8.3	8.3
As	0.6	0.6
Co	2.9	3.0
Ni	10.6	11
Sb	0.8	0.8
Pb	1.7	0.9
Total fatty acid intake (g /head/day)		
C14:0	0.99	0.99
C16:0	19.72	19.16
C16:1	0.41	0.46
C17:0	0.59	0.52
C18:0	4.25	4.19
C18:1 trans	0.35	0.30
C18:1 cis-9	26.40	25.04
C18:2 trans	0.08	0.19
C18:2 cis	25.27	26.36
C18:3n-6	0.27	0.27
C18:3n-3	0.64	0.61
C20:5n-3(EPA)	3.56	3.78
C20:2	0.30	0.25
C24:0	0.32	0.30

Enzymes activities determination

Enzymes activities were determined in blood plasma and milk by spectrophotometer (HITACHI U2000). The assayed enzymes were 1) Catalase (CAT; EC 1.11.1.6) assayed by the method of Beer and Sizer (1952). One unit of CAT is defined as the amount of enzyme which liberates half the peroxide oxygen from a H_2O_2 solution in 100 s at 25 °C.; 2) Lactoperoxidase (LPO; EC 1.11.1.7) by the method of Keesey (1987); 3) Glutathione *S*-transferase (GST; EC 2.5.1.18) spectrophotometrically at 340 nm by monitoring the rate of 1-chloro-2, 4 dinitrobenzene conjugation with glutathione as described by Habig *et al.*(1974) and the activity was expressed as nmol GSH consumed/min/mg protein.; 4) Glutathione reductase (GR; EC 1.6.4.2) in the hemolysate according to the method described by Mavis and stellwagen (1968) and expressed as nmol NADPH oxidized/min/mg protein.; 5) Glutathione peroxidase (seleno-dependent glutathione peroxidase (GSH-Px; EC 1.11.1.9) by the method of Wendal (1980) in which available GSH-Px catalyses the oxidation of reduced GSH by hydrogen peroxide. In the presence of GSH reductase and NADPH, oxidized GSH is converted to its reduced form with concomitant oxidation of NADPH to $NADP^+$. Oxidation of NADPH was measured by the decrease in the absorbance at 340 nm in a spectrophotometer.; 6) Superoxide dismutase (SOD; EC 1.15.1.1) by the method of McCord and Fridovich (1969) which consists of the inhibition of nitroblue tetrazolium (NBT) reduction with xanthine oxidase used as a superoxide generator. One unit of SOD activity is defined as the amount of protein that inhibits the rate of NBT reduction by 50 %.; and 7) Protein concentration by Bradford method (1976). All enzymes' activity was expressed as units per ml and units per mg of protein.

Preparation and analysis samples for Fatty acids

Diet and rumen; samples preparation

For FA methyl ester from samples of diets and rumen; the method described by O'Fallon *et al*. (2007) was followed. Grind samples in (1-1.5mm) or freeze dried rumen samples. In screw cap tube put 1g dry diet sample or 0.5 g freeze dried rumen sample plus 1

ml of internal stander C13:0 (0.5mg of C13:0/ml of MeOH) plus 0.7 ml of (10*N* KOH in water) plus 5.3 ml of MeOH. Incubate the tube at 55º C for 1.5 hr (water bath). Hand shaking for 5 sec. every 20 min. Put the samples in tap water to back to room temperature, adding 0.58 ml of (24*N* H_2SO_4), mixed by inversion until precipitated K_2SO_4, also incubate the tube at 55º C for 1.5 hr. Hand shaking for 5 sec every 20 min, back the samples in tap water to back to room temperature, adding 3 ml Hexane and vortex-mixed for 5 min, centrifuge for 5 min in a tabletop. In a GC vial: Take the hexane layer (containing FAME) placed at -20 º C for GC analysis.

For FA methyl ester from *Chlorella*, we follow the method described by Laurens *et al*. (2012). Approximately 15 mg Algae lyophilized powder are weighed in 100 mL glass tubes (screw cap with septum), adding of 0.2 mL Chloroform/ Methanol (2:1 v/v) , adding of 0.3 mL HCl /Methanol (5% v/v) , adding of 200 µl Hexane containing 250 µg of $C_{13:0}$-FAME internal Standard (Powder 250 µg in 200 µL Hexane). For 60 min in water bath at 85°C then cooling at room temperature. Extraction of FAMES by adding of 1 mL Hexane and 2 hours at room temperature. For concentration: By the help of a glass-pipette transferring the supernatant in a new Eppendorf, 800 µL of the supernatant is transferred in a new glass vial (50 mL – not restricted), placed in a water bath and evaporated with nitrogen at 35 °C. Resolution by adding of 100 µL Hexane then by the help of a glass-pipette immediately transferred to an Inlet and glass vials (storage at -20°C).

Blood plasma Samples preparation

Lipids were extracted from plasma according to the method described by Bondia- Pons *et al*. (2004). One hundred microlitres plasma samples were saponified in PTFE screw-capped Pyrex tubes containing 20 µg tridecanoic acid, by adding 1ml sodium methylate (0·5% w/v) and heating to 100 °C for 15 min. After cooling to 25 °C, samples were esterified with 1ml boron trifluoride-methanol reagent (also at 100 °C) for 15 min. Once the tubes were cooled, FAME were isolated by adding 500 µl n-hexane. After shaking for 1min, 1ml of a saturated sodium chloride solution was added. Finally, the tubes were centrifuged for 8 min at 2200 g. After drying with anhydrous sodium sulphate, the clear n-hexane top layer was transferred into a vial and used directly for chromatographic determination.

Milk samples preparation

A 17 ml milk sample was transferred into a separately funnel, where 30 ml of isopropanol were added. After vigorous shaking, 22.5 ml hexane was added and the mixture was shaken for another 3 min. The mixture was then centrifuged at 4000 rpm (2520×*g*) for 5 min at 5°C and the upper layer was transferred to a second separately funnel. The lower layer was extracted twice with 22.5 ml hexane and the supernatants were pooled with the previous hexane layer. After addition of 15 ml of 0.47 M aqueous Na_2SO_4 the hexane layer was collected into a flask and evaporated with a rotary evaporator at 30 °C.

For FA methyl esters of milk in 40 mg of lipid, 2 ml hexane was added, followed by 40 μl of methyl acetate. After vortex, 40 μl methylation reagent (1.75 ml methanol/0.4 ml of 5.4 mol/l sodium methylate) was added. The mixture was vortexed and allowed to react for 10 min, then 60 μl of termination reagent (1 g oxalic acid/30 ml diethylether) was added. The sample was then centrifuged for 5 min at 2400 × g at 5 °C and the liquid layer was removed and used directly for chromatographic determination. Fatty acid methyl esters were determined by gas chromatography using a Perkin Elmer Autosystem XL gas chromatograph equipped with an Omegawax 320 capillary column (30 m × 0.32 mm i.d. with 0.25 μm film thickness; Supelco, Sigma–Adrich Co., USA). A flame ionization detector temperature was set at 220 °C and the chromatographic analysis involved a temperature programmed run starting at 50 °C and held for 2 min and then followed by a step up ramp of 4 °C/min to 220 °C and held for 20 min. Helium was used as the carrier gas with a linear velocity set at 30 cm/s. Each peak was identified and quantified using a 37 component FAME mix standard (Supelco, Sigma–Adrich Co., USA). CLA in particular was identified using a conjugated linoleic acid methyl ester standard (Sigma–Adrich Co., USA). A heptadecanoic (C17:0) FA methyl ester (Larodan, Sweden) was added to the methylated milk fat samples prior to GC analysis and was used as an internal standard for the chromatographic analysis. The fatty acid composition of milk fat is expressed as percent amount (%) of each individual FA per total fatty acids detected (Tsiplakou *et al*., 2006).

The different groups of FA were defined as follows: short chain fatty acids (SCFA) = C6:0 +C8:0 +C10:0 +C11:0, medium chain fatty acids (MCFA) = C12:0 +C13:0 +C14:0

+C15:0, long chain fatty acids (LCFA) = C16:0 +C18:0 +C20:0 +C22:0 +C23:0 +C24:0, poly-unsaturated fatty acids (PUFA) = CLA + C18:2n6c +C18:2n6t +C18:3n3c +C18:3n6c +C20:2 +C20:3n3c +C20:3n6c +C20:4 +C20:5 +C22:2, mono-unsaturated fatty acids (MUFA)=C14:1+C15:1+C16:1+C17:1+C18:1+C20:1, saturated/unsaturated (S/U) = (SCFA +MCFA + LCFA) /(PUFA + MUFA), CLA: C18:2 cis-9, trans-11. The atherogenicity index was defined as (AI): (C12:0 +4C14:0 +C16:0)/U described by Ulbricht and Southgate (1991). The Δ−9 desaturase activity indexes were calculated by the following ratios: C14:1/C14:0 and, C18:1/C18:0.

Statistical analysis

The experimental data were subjected to statistical analysis with SPSS 17.0.2 Statistical package (SPSS Inc., Chigaco, IL, USA). The body weight, the milk chemical composition, the enzyme activity, and FA of milk and blood plasma were analyzed using a general linear model (GLM) for repeated measures analysis of variance (ANOVA) with dietary treatments (CON and ALG) and sampling time as fixed effects and their interactions (D*T) according to the model: Yijk = μ+ Ai + Bj + Ai*Bj +eijk, where Yijk values of observation; μ= general mean; Ai is the effect of the diet (CON vs. ALG); Bj is the effect of sampling time; Ai*Bj is the interaction between the diet and sampling time; and eijk is the residual error. Comparison of means was performed using the Bonferonni post hoc test. A value of $P \leq 0.05$ was considered significant. To statistical analysis of rumen FAs profile, we used compare means with independent t-test. A value of $P \leq 0.05$ was considered significant

3.1.1.3 Results

Diets and feed intake

Table (3.1.1.1) shows the chemical composition of wheat straw, alfalfa hay, concentrate mixture and *C. pyrenoidosa*. The *C. pyrenoidosa* which used in the current study was high in protein (57%), and contained considerable amounts of minerals. Table (3.1.1.2) presents the average daily of dry matter, nutrients, minerals, and FA intake. From FA intake (g/head/ day), we noted that the major FA intake was linoleic acid (C18:2n-6) and there was a comparable intake in CON compared with the ALG (25.27 vs. 26.36 respectively). The

second most abundant dietary FA was oleic acid (18:1 cis-9), with 26.40 and 25.04 (g/head /day) for CON and ALG, respectively.

Effects of dietary *Chlorella pyrenoidosa* supplementation on goat's performance

Table (3.1.1.3) presents the effect of experimental treatment on body weight, and milk yield and composition. The average milk yield (g/d) was 1733 and 1708 for the CON and ALG groups respectively, and the average fat corrected milk yield (g/d) 1555 and 1540 respectively without any significant difference ($P>0.05$) between the two groups. The average milk fat percentages for CON and ALG groups were 3.36% and 3.44% respectively without significant difference ($P>0.05$) between the two groups. However, the average milk protein content of the whole experimental period was higher ($P>0.05$) in CON group compared to that in ALG group (3.00% vs. 2.77%). On the other hand, the sampling time affected significantly the body weight ($P<0.001$), milk yield, fat corrected milk yield ($P<0.001$ and $P< 0.01$ respectively), fat ($P<0.001$), and lactose content ($P<0.001$) and daily fat, protein and lactose yield ($P<0.001$).

Table 3.1.1.3 Effect of experimental treatment on body weight, milk yield and milk composition in both groups (CON and ALG) of goats.

Item	Diets[1]			Sampling time					Significance			
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 7 (n=16)	Day 14 (n=16)	Day 21 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
Body weight, kg	50.16	48.32	4.74	47.40^{a}	48.80^{b}	49.80^{bc}	50.00^{c}	50.20^{bc}	0.340	NS	***	NS
Milk yield, g/d	1733	1708	337	1711^{ac}	1832^{b}	1774^{ab}	1699^{ac}	1585^{c}	31	NS	***	NS
Fat corrected milk yield(4%), g/d	1555	1540	271	1590^{abc}	1649^{a}	1547^{b}	1511^{bc}	1440^{cd}	26	NS	**	NS
Chemical composition												
Fat (%)	3.36	3.44	0.337	3.61^{a}	3.38^{ab}	3.23^{bc}	3.34b^{ac}	3.45^{a}	0.069	NS	***	NS
Protein (%)	3.00	2.77	0.227	3.01	2.84	2.87	2.82	2.88	0.072	NS	NS	NS
Lactose (%)	4.44	4.38	0.099	4.38^{abc}	4.50^{a}	4.29^{b}	4.40^{c}	4.47^{ac}	0.059	NS	***	NS
Fat, g/d	56.80	56.20	9.39	56.75^{ac}	60.75^{b}	55.81^{cd}	55.43^{ad}	53.75^{ac}	1.21	NS	***	NS
Protein, g/d	51.08	46.98	9.51	51.63^{ab}	51.84^{a}	50.25^{ac}	47.13^{b}	44.58^{b}	1.34	NS	***	NS
Lactose, g/d	77.16	75.13	14.97	76.85^{ac}	82.26^{b}	76.00^{a}	74.79^{ac}	70.84^{c}	1.35	NS	***	NS

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10 g *C. pyrenoidosa/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference. Values are reduced model Bonferroni.

$^{a–c}$ Within a row, different superscripts indicate significant differences among sampling time ($P < 0.05$).

*** P< 0.001; ** P < 0.01; * P < 0.05; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Effects of dietary *Chlorella pyrenoidosa* supplementation on blood plasma and milk enzymes activities

Blood plasma enzymes activity

The statistical evaluation of the goat's blood plasma enzymes activity (in units per ml of plasma and in units per mg of protein) is presented in Table 3.1.1.4. The GR and SOD activities, expressed as units/ml plasma were significantly higher ($P<0.05$ and $P<0.01$ respectively) in ALG compared with those of CON during the experiment. In addition, SOD was significantly affected by sampling time ($P<0.01$). Likewise, SOD showed significant interaction between diets and sampling time ($P<0.001$) during the experiment. The blood plasma protein concentration was significantly higher ($P<0.01$) in the ALG group compared to the CON group during the experiment. The GR activity, expressed as units/mg protein, was significantly lower ($P<0.05$) in the ALG group compared with that in CON group, while the SOD did not show any significant difference ($P>0.05$) between the two groups during the experimental period.

Milk enzymes activity

Table 3.1.1.5 shows the statistical evaluation of the goat's milk enzymes activity in units per ml of milk and in units per mg of protein. First, as units per ml milk: There were no significant differences ($P>0.05$) between the two groups during the experimental period in milk CAT, LPO, GR, and SOD activities. On the other hand, the milk GSH-Px activity was significantly lower ($P<0.01$) in ALG, that in CON group during the experimental period. There was no significant difference ($P>0.05$) between the two groups in the milk protein concentration during the whole experimental period. Second, as units per mg of protein: there was no significant differences ($P>0.05$) between the two groups during the experimental period in all milk enzymes activities (Table 3.1.1.5). The sampling time affected significantly CAT, LPO, and SOD activities ($P<0.001$, $P<0.01$, $P<0.05$ respectively), and the interaction between diet and sampling time was significant ($P<0.05$) only for milk protein concentration.

Table 3.1.1.4 Goats blood plasma enzymes activity (in Units/ ml, Units/ mg protein) and protein concentration (in mg/ml) in both groups (CON and ALG).

Item	**Diets[1]**			**Sampling time**				**Significance**		
	CON (n=8)	**ALG (n=8)**	**SEM[2]**	**Day 1 (n=16)**	**Day 14 (n=16)**	**Day 28 (n=16)**	**SEM[2]**	**D[3]**	**T[4]**	**D*T[5]**
GST U/ ml	0.117	0.104	0.021	0.104	0.128	0.100	0.016	NS	NS	NS
GST U/ mg protein	0.017	0.010	0.004	0.016	0.016	0.010	0.002	NS	NS	NS
GR U/ ml	0.046^{A}	0.051^{B}	0.002	0.048	0.048	0.050	0.003	*	NS	NS
GR U/ mg protein	0.006^{A}	0.005^{B}	0.000	0.007^{a}	0.006^{ab}	0.005^{b}	0.000	*	*	NS
GSH-Px U/ ml	0.146	0.167	0.009	0.154	0.160	0.157	0.010	NS	NS	NS
GSH-Px U/ mg protein	0.020	0.017	0.001	0.022^{a}	0.019^{b}	0.015^{b}	0.002	NS	**	NS
SOD U/ ml	8.017^{A}	17.662^{B}	1.492	9.542^{a}	14.989^{b}	13.987^{ab}	1.340	**	**	***
SOD U/ mg protein	1.190	1.666	0.259	1.425	1.578	1.281	0.245	NS	NS	**
Protein mg/ml	7.63^{A}	10.47^{B}	0.638	7.39^{a}	8.97^{ab}	10.79^{b}	0.699	**	***	NS

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. pyrenoidosa/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference. Values are reduced model Bonferroni.

[A-B] Within a row, different superscripts indicate significant differences between diets (P<0.05).

[a–b] Within a row, different superscripts indicate significant differences among sampling time (P<0.05).

*** P<0.001; ** P <0.01; * P< 0.05; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Table 3.1.1.5 Goats milk enzymes activity (in Units/ ml, Units/ mg protein) and protein concentration (in mg/ml) in both groups (CON and ALG).

Item	**Diets[1]**			**Sampling time**				**Significance**		
	CON (n=8)	**ALG (n=8)**	**SEM[2]**	**Day 1 (n=16)**	**Day 14 (n=16)**	**Day 28 (n=16)**	**SEM[2]**	**D[3]**	**T[4]**	**D*T[5]**
CAT U/ ml	245.50	239.93	23.1	281.43^{a}	210.41^{ab}	236.32^{b}	19.198	NS	***	NS
CAT U/ mg protein	31.22	29.77	4.67	36.67 ^{a}	25.50 ^{ab}	29.32 ^{b}	4.009	NS	*	NS
LPO U/ ml	0.037	0.029	0.01	0.031^{ab}	0.044^{a}	0.025^{bc}	0.005	NS	**	NS
LPO U/ mg protein	0.005	0.004	0.00	0.004^{ab}	0.005^{a}	0.004^{b}	0.001	NS	*	NS
GR U/ ml	0.108	0.091	0.02	0.115	0.099	0.085	0.032	NS	NS	NS
GR U/ mg protein	0.013	0.012	0.00	0.015	0.011	0.011	0.004	NS	NS	NS
GSH-Px U/ ml	0.308^{A}	0.245^{B}	0.01	0.294	0.283	0.253	0.029	**	NS	NS
GSH-PxU/mg	0.039	0.031	0.00	0.038	0.034	0.034	0.004	NS	NS	NS
SOD U/ ml	120.96	124.61	15.6	89.06^{a}	132.32^{ab}	146.97^{b}	15.05	NS	*	NS
SOD U/ mg protein	15.67	18.68	2.59	16.74	16.11	18.68	2.518	NS	NS	NS
Protein mg/ml	8.10	8.53	0.89	7.76	8.97	8.23	0.672	NS	NS	*

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. pyrenoidosa/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference. Values are reduced model Bonferroni.

$^{A-B}$ Within a row, different superscripts indicate significant differences between diets ($P < 0.05$).

$^{a–c}$ Within a row, different superscripts indicate significant differences among sampling time ($P < 0.05$).

*** $P< 0.001$; ** $P < 0.01$; * $P < 0.05$; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Effects of dietary *Chlorella pyrenoidosa* supplementation on rumen, blood plasma and milk fatty acid profile

Concentration of fatty acids in rumen, blood plasma and milk samples

In the rumen FA profile with addition 10 g of *C. pyrenoidosa*/kg concentrates Table 3.1.1.6, there was not a lot of significant changes observed but the results indicated that, there was increase of accumulation of C18:0 concentration in ALG compared with that of CON (40.36 vs. 29.54 respectively). Also, there was a clear tend to decrease in the C18 intermediates (C18:1 trans, C18:1 cis9, C18:2n-6 trans and C18:2n-6 cis concentrations) in ALG compared with that of CON without significant difference compared with those of CON. An incomplete biohydrogenation of PUFA concentration in the rumen is also suggested by a lack of increase in stearic acid C18:0 concentration in ALG group. Also, it was noted that the tendency of decrease of C18:3n-3 concentration leaded to decrease of VA and CLA concentrations in ALG group. On the other hand, there an increase on SCSFA and LCSFA concentrations while significantly decreased ($P<0.05$) MUFA concentration and C14:1/C14:0 compared with those of CON.

In plasma FA profile of ALG group there was a decrease ($P<0.05$) in C18:2n-6 trans, C18:3n-6, C20:1, C21:0, C20:2, and C20:4 concentrations. On the other hand, there was an increase in C18:0 concentration but without significant difference compared with that of CON group (Table 3.1.2.7). From rumen and plasma FA, we noted an increase of accumulation of C18:0 concentration in ALG compared with that of CON.

In milk results, the daily incorporation of 10 g *C. pyrenoidosa* /kg concentrate mixture decreased ($P< 0.05$) some of the SCFA (C6:0 , C8:0, and C10:0), MCFA (C12:0 and C15:0) and LCFA (C18:0 and C20:0) concentrations compared with those of CON, while the C4:0 , C14:0 and C16:0 concentrations tend to increase ($P>0.05$) without significant difference between groups. Also, in milk of ALG group, a significant increase ($P<0.05$) was observed in C18:1 trans concentration compared with CON during the experiment (Table 3.1.2.8). In milk of ALG group, the LCFA and MUFA concentrations were increased and

Table 3.1.1.6 Mean (±S.E) concentration of fatty acids (% of total fatty acids) in rumen of CON and ALG groups.

	Diets[1]		
Fatty acids	**CON (n=3)**	**ALG (n=3)**	**Significance**
C4:0	0.42[a] ±0.422	2.40[b] ±0.561	*
C6:0	0.22 ±0.220	0.73 ±0.341	NS
C8:0	0.04 ±0.040	0.08 ±0.041	NS
C10:0	0.21 ±0.039	0.16 ±0.034	NS
C11:0	0.27 ±0.067	0.19 ±0.015	NS
C12:0	9.16 ±1.470	5.95 ±1.131	NS
C14:0	1.87 ±0.443	1.48 ±0.088	NS
C14:1	4.58 ±1.099	2.92 ±0.257	NS
C15:0	2.08 ±0.458	1.54 ±0.071	NS
C15:1	0.18 ±0.013	0.17 ±0.006	NS
C16:0	19.19 ±1.639	17.68 ±0.142	NS
C16:1	0.68 ±0.103	0.47 ±0.024	NS
C17:0	0.80 ±0.072	0.69 ±0.028	NS
C18:0	29.54 ±4.139	40.36 ±1.739	NS
C18:1 trans	1.23 ±0.316	1.21 ±0.220	NS
C18:1 cis -9	10.47 ±1.340	9.12 ±1.171	NS
C18:2n-6 trans	0.35 ±0.117	0.15 ±0.019	NS
C18:2n-6 cis (LA)	6.25 ±0.769	4.92 ±0.301	NS
C20:0	0.34 ±0.024	0.32 ±0.066	NS
C18:3n-3(LNA)	0.87 ±0.111	0.78 ±0.044	NS
CLA	3.82 ±0.539	2.63 ±0.332	NS
20:3n-6	0.21 ±0.058	0.26 ±0.011	NS
C18:3n-3(LNA)	0.70 ±0.158	0.58 ±0.022	NS
22:2	0.21 ±0.045	0.18 ±0.008	NS
20:5	0.25 ±0.065	0.16 ±0.014	NS
24:0	0.33 ±0.066	0.34 ±0.014	NS
24:1	0.47 ±0.040	0.53 ±0.026	NS
VA	4.78 ±0.600	3.70 ±1.189	NS
C18:1 trans-10	0.47 ±0.147	0.31 ±0.039	NS
SCSFA	1.16 ±0.312	3.57 ±0.921	NS
MCSFA	13.11 ±1.953	8.97 ±1.233	NS
LCSFA	49.40 ±3.449	58.69 ±1.672	NS
PUFA	12.67 ±1.104	9.65 ±0.225	NS
MUFA	17.13 ±0.611	13.89[b] ±0.957	*
S/U	2.15[a] ±0.162	3.04[b] ±0.156	*
AI	1.20 ±0.084	1.26 ±0.045	NS
C14:1/C14	2.46[a] ±0.101	1.97[b] ±0.126	*
C16:1/C16	0.03 ±0.003	0.03 ±0.001	NS
C18:1/C18	0.04 ±0.014	0.03 ±0.007	NS
CLA/VA	0.80 ±0.045	0.91 ±0.372	NS

Diets[1]: CON= control group without micro algae; ALG=microalgae group with 10g micro algae *C. pyrenoidosa* / kg of concentrate mixture.

Means of fatty acids with different letters (a, b) between groups CON and ALG, differ significantly ($P \leq 0.05$). *** P<0.001; ** P<0.01; * P<0.05; NS: No significance.

Table 3.1.1.7 Fatty acids concentrations (% of total fatty acids) of lipids in goats blood plasma in both groups (CON and ALG).

Fatty acids	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
C14:0	0.33	0.38	0.030	0.37	0.35	0.35	0.052	NS	NS	NS
C15:0	0.53	0.50	0.012	0.48^{a}	0.51^{ab}	0.54^{c}	0.021	NS	*	NS
C15:1	0.08^{A}	0.22^{B}	0.029	0.10	0.18	0.18	0.050	**	NS	NS
C16:0	14.60	14.62	0.216	15.09	13.95^{b}	14.79^{ab}	0.374	NS	**	NS
C16:1	0.89	0.78	0.040	0.88	0.82	0.82	0.069	NS	NS	*
C17:0	1.07	0.98	0.037	1.14^{a}	0.89^{b}	1.05^{ab}	0.064	NS	**	*
C17:1	0.28	0.28	0.024	0.29	0.28	0.27	0.042	NS	NS	NS
C18:0	24.42	25.10	0.426	25.15	24.65	24.48	0.737	NS	NS	NS
C18:1 trans	0.61	0.52	0.032	0.59	0.57	0.54	0.056	NS	NS	NS
C18:1 cis-9	14.64	14.73	0.708	13.37	16.16	14.53	1.226	NS	NS	NS
C18:2n-6 trans	0.63^{A}	0.47^{B}	0.043	0.37^{a}	0.72^{b}	0.57^{b}	0.074	*	***	***
C18:2n-6 cis(LA)	32.03	32.33	0.663	32.82	31.39	32.32	1.149	NS	NS	NS
C18:3n-6	0.34^{A}	0.29^{B}	0.018	0.24^{a}	0.39^{b}	0.32^{ab}	0.031	*	***	NS
C18:3n-3(LNA)	1.13	1.17	0.030	1.12^{ac}	1.07^{ab}	1.25^{c}	0.052	NS	**	NS
C20:1	0.54^{A}	0.23^{B}	0.048	0.20^{a}	0.47^{b}	0.48^{b}	0.083	***	**	NS
C21:0	0.32^{A}	0.21^{B}	0.035	0.22	0.34	0.23	0.061	*	NS	*
C20:2	0.43^{A}	0.33^{B}	0.029	0.32	0.43	0.39	0.050	*	NS	NS
C20:3n-6	0.49	0.51	0.036	0.50	0.50	0.50	0.062	NS	NS	NS
C20:3n-3+C22:1	5.29	4.99	0.134	5.41	4.91	5.10	0.232	NS	NS	NS
C20:4	0.73^{A}	0.30^{B}	0.075	0.29^{a}	0.86^{b}	0.41^{a}	0.129	***	***	**
C22:2	0.54	0.58	0.024	0.65^{a}	0.48^{b}	0.55^{ab}	0.042	NS	**	*

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. pyrenoidosa* / kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

Values are reduced model Bonferroni.

$^{A–B}$ Within a row, different superscripts indicate significant differences between diets (P< 0.05).

$^{a–c}$ Within a row, different superscripts indicate significant differences among sampling time (P< 0.05).

*** P< 0.001; ** P < 0.01; * P < 0.05; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Table 3.1.1.8 Concentration of fatty acids (% of total fatty acids) in milk fat of CON and ALG groups.

Fatty acids	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
C4:0	3.01	3.38	0.267	2.85	3.48	3.25	0.33	NS	NS	NS
C6:0	3.40	3.29	0.143	3.34	3.32	3.36	0.18	NS	NS	NS
C8:0	3.70^{A}	3.15^{B}	0.216	3.65	3.18	3.43	0.27	*	NS	NS
C10:0	9.95	9.15	0.521	10.14	8.83	9.69	0.64	NS	NS	NS
C12:0	3.30	3.14	0.221	3.36	2.92	3.39	0.27	NS	NS	NS
C14:0	7.79	8.06	0.509	7.88	7.69	8.20	0.62	NS	NS	NS
C14:1	0.33	0.37	0.039	0.30	0.42	0.33	0.05	NS	NS	NS
C15:0	0.92 ^{A}	0.83 ^{B}	0.041	0.83	0.88	0.91	0.05	*	NS	NS
C15:1	0.30	0.26	0.026	0.26	0.33	0.25	0.03	NS	NS	NS
C16:0	24.09	25.29	1.169	24.77	24.59	24.71	1.43	NS	NS	NS
C16:1	0.84 ^{A}	0.62 ^{B}	0.041	0.80^{a}	0.64^{b}	0.76^{ab}	0.05	***	**	**
C17:1	0.19	0.21	0.016	0.17	0.20	0.22	0.02	NS	NS	NS
C18:0	12.72	12.17	0.629	12.98^{a}	13.58^{a}	10.78^{b}	0.77	NS	*	NS
C18:1 trans	0.68 ^{A}	0.87 ^{B}	0.082	0.83	0.80	0.71	0.10	*	NS	NS
C18:1 cis -9	20.38	21.25	1.466	19.20	21.15	22.10	1.80	NS	NS	NS
C18:2n-6 trans	0.34	0.35	0.036	0.32	0.35	0.36	0.05	NS	NS	**
C18:2n-6 cis(LA)	2.67	2.68	0.284	2.60	2.80	2.63	0.35	NS	NS	NS
C18:3n-6	0.37	0.33	0.117	0.40	0.32	0.33	0.14	NS	NS	NS
C20:0	0.14	0.13	0.006	0.14	0.14	0.13	0.01	NS	NS	*
C18:3n-3(LNA)	0.30	0.28	0.029	0.30	0.27	0.29	0.04	NS	NS	NS
C20:3n-3+22:1	0.10	0.08	0.026	0.09	0.08	0.09	0.03	NS	NS	***

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. pyrenoidosa* / kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

Values are reduced model Bonferroni.

$^{A–B}$ Within a row, different superscripts indicate significant differences between diets (P< 0.05).

$^{a–d}$ Within a row, different superscripts indicate significant differences among sampling time (P < 0.05).

*** P< 0.001; ** P < 0.01; * P < 0.05; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Table 3.1.1.9 Fatty acids groups, CLA (% of total fatty acids), S/U and AI values of milk fat of CON and ALG groups.

Fatty acids	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
SCFA	20.06	18.96	0.626	19.99	18.82	19.73	0.766	NS	N	N
MCFA	12.00	12.03	0.652	12.07	11.48	12.50	0.798	NS	N	N
LCFA	36.94	37.59	1.29	37.88	38.30	35.62	1.58	NS	N	N
PUFA	4.66	4.47	0.373	4.51	4.57	4.61	0.457	NS	N	N
MUFA	24.91	25.82	1.465	23.86	25.69	26.55	1.794	NS	N	N
S/U	2.55	2.29	0.311	2.76	2.30	2.20	0.381	NS	N	N
AI	2.19	2.02	0.312	2.38	1.96	1.99	0.382	NS	N	N
CLA	0.89	0.76	0.088	0.80	0.76	0.91	0.108	NS	N	N
VA	2.18	2.26	0.259	2.31	2.16	2.18	0.318	NS	N	N
C18:1 trans-10	1.42	1.09	0.338	1.63	1.14	1.00	0.413	NS	N	N

Table 3.1.1.10 Comparison of *Δ*9-desaturase activity indexes in milk fat of CON and ALG groups.

Item	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
C14:1/C14	0.05	0.05	0.007	0.04	0.06	0.04	0.008	NS	NS	N
C16:1/C16	0.04 [A]	0.03 [B]	0.002	0.03	0.03	0.03	0.003	***	NS	*
C18:1/C18	0.05 [A]	0.07 [B]	0.008	0.06	0.06	0.07	0.010	*	NS	N
CLA/VA	0.42	0.34	0.043	0.34	0.38	0.43	0.053	NS	NS	N

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. vulgaris/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

Values are reduced model Bonferroni.

[A-B] Within a row, different superscripts indicate significant differences between diets ($P < 0.05$).

[a–c] Within a row, different superscripts indicate significant differences among sampling time ($P< 0.05$).

[***] $P< 0.001$; [**] $P < 0.01$; [*] $P < 0.05$; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

there was a tendency to decrease SCFA and PUFA concentrations without any significant difference. Also, the value of AI tended to decrease but not significantly in ALG group compared to CON (Table 3.1.2.9). On the other hand, a significant increase ($P<0.05$) in *C*18:1/*C*18:0 was observed in ALG milk compared with the CON (Table 3.1.2.10).

3.1.1.4 Discussion

Diets and feed intake

Table 3.1.1.1 shows the chemical composition of wheat straw, alfalfa hay, concentrate mixture and *C. pyrenoidosa*. The *C. pyrenoidosa* which used in the current study was high in protein (57%), and contained considerable amounts of minerals. It was close to the chemical composition of *C. pyrenoidosa* reported by Phang (1992).

Table 3.1.1.2 presents the average daily of dry matter, nutrients, minerals, and FA intake. From these data, it can be seen that the intake of most nutrients was comparable between the two groups. Inconsistent results have been observed with micro algae supplementation and longer trials (Papadopoulos *et al*., 2002; Reynolds *et al*., 2006). Although in dairy cows micro algae supply reduced feed intake (Franklin *et al*., 1999; Boeckaert *et al*., 2008b) in this experiment no difference in feed intake was observed.

Effects of dietary *Chlorella pyrenoidosa* supplementation on goat's performance

Table 3.1.1.3 presents the effect of experimental treatment on body weight, and milk yield and composition. There was no significant difference in the average milk yield or the average fat corrected milk yield between the two groups. The average milk fat percentages for CON and ALG groups were 3.36 % and 3.44 % respectively without significant difference ($P>0.05$) between the two groups. However, the average milk protein content of the whole experimental period was higher ($P>0.05$) in CON group compared to that in ALG group (3.00% vs. 2.77%). This result agrees with that of Franklin *et al*. (1999) who were the first one used micro algae in dairy cows. The study demonstrated a tendency for decreased milk protein content in the algae group (37.7 vs. 32.8 g/kg). The lactose content of milk did not show any significant difference between the two groups. The micro algae

supplementation did not negatively affect the milk fat content which is an important parameter in cheese yield for the dairy sheep breeders, because ovine milk is mostly transformed into cheese while the opposite happened in dairy cows (Donovan *et al*., 2000; Boeckaert *et al*., 2008b).

On the other hand, the sampling time affected significantly the body weight ($P<0.001$), milk yield, fat corrected milk yield ($P<0.001$ and $P<0.01$ respectively), fat ($P<0.001$), and lactose content ($P< 0.001$) and daily fat, protein and lactose yield ($P<0.001$). This result of increase body weight and decrease milk yield, fat corrected milk yield, daily fat, protein and lactose yield in both groups (CON and ALG) throughout the experimental period follows a normal trend of a lactation curve (Arguello *et al*., 2006).

Effects of dietary *Chlorella pyrenoidosa* supplementation on blood plasma and milk enzymes activities

Chlorella recently gained attention because of numerous reports on its health benefits for antioxidant, immune activation *in vitro* and *in vivo*. However, the antioxidant effect of *Chlorella* had yet to be demonstrated in a human study. The potential antioxidant capacity of *Chlorella* has been attributed to the effect of specific ingredients in *Chlorella*, such as chlorophylls, antioxidant vitamins, and phenol compounds (Table 3.2.6.1).

Blood plasma enzymes activity

The statistical evaluation of the goat's blood plasma enzymes activity (in units per ml of plasma and in units per mg of protein) is presented in Table 3.1.1.4. The SOD activity in blood plasma was significantly higher ($P<0.01$) in ALG compared with that of CON during the experimental period. The higher SOD activity in ALG indicates that *C. pyrenoidosa* had an antioxidant effect like SOD. Our results are in agreement with those of Lee *et al*. (2003) who reported that *Chlorella* supplementation decreased superoxide anion production in plasma of mice because it increased SOD activity. The same results confirmed in human plasma by Lee *et al*. (2010) who reported that *Chlorella* supplement exhibits antioxidant activity decreasing ROS and increasing the activity of SOD. The most important substance in

Chlorella seems to be a β-1,3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore *et al.,* 2006b). The increase of activities for SOD and GSH-Px in ALG may be related to these compounds in *C. pyrenoidosa* which cotain appreciable amounts of bioactive compounds (Table 2.3.6.1).

Glutathione reductase activity in blood plasma was significantly higher ($P<0.05$) in ALG compared with that of CON during the experimental period. Vijayavel *et al*. (2007) state that the GR activity represents more glutathione available for quenches ROS and as a cofactor for antioxidant enzymes activity. There is much evidence of how the vitamins modulate intracellular signals and act as antioxidants. Vitamin C is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. The antioxidant enzymes metabolize oxidative toxic intermediates and require micronutrient cofactors such as Se, Fe, Co, Zn, and Mn for optimum catalytic activity.

The blood plasma protein concentration was significantly higher ($P< 0.01$) in ALG compared to CON during the experiment and there was significant difference ($P< 0.001$) among sampling time. *Chlorella pyrenoidosa* is the only known natural substance to raise albumin levels. High albumin levels have been shown to profoundly extend the life span of cells *in vitro* and to prevent the mutation of cancer lines. Albumin is the most abundant fraction (52- 62%) of the blood protein in goats (Zubcic, 2001). Su-Ching *et al*. (2012) found that the incorporation of 4 or 8% *Chlorella* in rats diets increased the albumin concentration in their blood plasma compared with that of the control group.

Milk enzymes activity

The antioxidant defiance system in micro algae includes besides carotenoids, α-tocopherol and ascorbic acid (Rao *et al.,* 1996; Malanga *et al.,* 1997; Rijstenbil, 2002). The GSH-Px activity in our experiment was significantly lower ($P<0.01$) in ALG compared with that of CON during the experiment (Table 3.1.1.5). This result of GSH-Px (0.308-0.245U/ml) is comparable with that of Wullepit *et al*. (2011) who reported that the level of GSH-Px in cow's milk was 0.077-0.110 U/ml.

In our results lactoperoxidase activity in milk was lower (P>0.05) in ALG compared with that of CON during the experimental period. In the present study LPO activity was lower than the values reported by Zapico *et al*. (1990) and Saad de Schoos *et al*. (1999) and close to the result which reported by Eyassu *et al*.(2004). Lactoperoxidase appears to be constitutively present in milk (Marshall *et al*., 1986; Fonteh *et al*., 2002; Isobe *et al*., 2009), although LPO activity in milk was up regulated by the lactoperoxidase system inoculation into mammary gland of cows (Isobe *et al*., 2009). However, little is known about factors regulating its expression and activity (Fragoso *et al*., 2009).

In our study, the range of results for LPO activity was 0.025 to 0.044 U/ml. Previous reports have indicated different levels of LPO activity in goat milk. Zapico *et al*. (1990) reported LPO activity of 1.55 U/ml in raw goat milk from Spain. The LPO activity of Creole goat milk was found to be 4.45 U/ml (Saad de Schoos *et al*., 1999). Eyassu *et al*. (2004) reported that the level of LPO activity in goat milk was 0.26- 0.79 U/ml. In Verata and Murciano-Granadina goat milk, Zapico *et al*. (1991) found the lowest values of LPO activity (0.03 and 0.20 U/ml, respectively) in the first 24 h after kidding, before an increase during the 75 d postpartum (0.82 and 3.47 U/ml, respectively). Nevertheless, in Verata goat milk, LPO values continued to rise until the end of lactation (1.44 U/ml, 150 d), while in Murciano-Granadina goat milk, a gradual decline until late lactation with an activity level of 2.07 U/ml (150 d) was described.

Effects of dietary *Chlorella pyrenoidosa* supplementation on rumen, blood plasma and milk fatty acid profile

Concentration of fatty acids in rumen, blood plasma and milk samples

In the rumen FA with addition 10g of *C. pyrenoidosa*/kg concentrates Table 3.1.1.6, there was increase of accumulation of C18:0 concentration in ALG compared with that of CON. Also, there was a clear tend to decrease in the C18 intermediates in ALG group without significant difference compared with those of CON. Also, it was noted that the tendency of decrease of C18:3n-3 concentration leaded to decrease of VA and CLA

concentrations in ALG group. On the other hand, there was increased on SCSFA and LCSFA concentrations while significantly decreased (P<0.05) MUFA concentration and C14:1/C14:0 compared with those of CON.

In plasma of ALG group there was a decrease (P<0.05) in C18:2n-6 trans, C18:3n-6, C20:1, C21:0, C20:2, and C20:4 concentrations. On the other hand, there was an increase in C18:0 concentration in ALG but without significant difference compared with that of CON group (Table 3.1.2.7). The same trend of the long chain fatty acids were observed in plasma and milk. This may due to the fact that forty to sixty per cent of milk fatty acids are long-chain (predominantly C18) derived from the diet and more than 90 % of these fatty acids in milk are of plasma origin, consistent with the demonstration of little fatty acid elongation in the mammary gland (Palmquist *et al*., 1969).

In milk results, the daily incorporation of 10 g *C. pyrenoidosa* /kg concentrate mixture decreased (P< 0.05) some of the SCFA (C6:0 , C8:0, and C10:0), MCFA (C12:0 and C15:0) and LCFA (C18:0 and C20:0) concentrations compared with those of CON, while the C4:0 , C14:0 and C16:0 concentrations tend to increase (P>0.05) without significant difference between groups (Table 3.1.2.8). Half of milk palmitic acid (16:0) comes from endogenous synthesis and the other half from the diet. Analogous results in dairy cows have been reported by Franklin *et al*. (1999) and Boeckaert *et al*. (2008b) when they examined the dietary use of micro algae *Schizochytrium sp.* The decrease in the proportion of short- and medium-chain FA (C6:0 to C12:0) with micro algae supplementation is in agreement with others (Donovan *et al*., 2000; Boeckaert *et al*., 2008b) who reported similar reductions in *de novo* FA synthesis in cows fed oil and/or micro algae. Additionally, the reduction on SCFA concentration in goat milk of ALG can be seen as a positive effect, because accumulated evidence shows a strong link between the intake of some SFA and the incidence of cardiovascular diseases (Hu *et al*., 2001; Mensink *et al*., 2003).

According to our results, C8:0 and C15:0 concentrations were (P< 0.05) decreased in ALG group compared with CON group. These results disagree with those of Moreno-Indias *et al*. (2014) who used 5 g of *C. pyrenoidosa* in dairy goats. It has been reported that the addition of micro algae caused a reduction on milk FA's with less than 16 C and an increase

in FA with more than 16 C (Toral *et al*., 2010), or a decrease in the ruminal outflow of C18:0 or an increase in the CLA content (Bichi *et al*. 2013), although the low amount of C. *pyrenoidosa* used in the present study, there was affect on some parameters like reduction in short and medium-chain saturates.

Also, in milk of ALG group, a significant increase ($P< 0.05$) was observed in C18:1 trans concentration compared with CON during the experiment (Table 3.1.2.8). The increase in C18:1 trans concentration production could play a protective role for bacteria, thus reducing membrane fluidity as a defense mechanism against lipophylic or toxic stress stimuli such as high concentration of PUFA (Bessa *et al*., 2000).

In milk of ALG group, the LCFA and MUFA concentrations were increased and there was a tendency to decrease SCFA and PUFA concentrations without any significant difference (Table3.1.2.9). The same results were observed by Moreno-Indias *et al*. (2014) who used *C. pyrenoidosa* in dairy goats and they reported that there was not significant difference in both PUFA and MUFA concentrations. The difference in PUFA concentration was not significant between ALG and CON because there was no difference in PUFA intake between the two groups. Tsiplakou *et al*. (2008) reported that increasing the supply of n-3 PUFA in the diet is one of the most important ways of improving the PUFA content of ruminant milk. The same trend of the changes in LCSFA and PUFA concentrations observed in rumen and milk in the present study.

Previous research showed that feeding micro algae (*Schizochytrium sp*) with high content of DHA (162 g/kg DM) to dairy cows (150 g/cow) may replace fish oil in dairy cow diets as a modifier for rumen biohydrogenation to increase milk CLA content (Abu Ghazaleh *et al*., 2009). Similar results have been reported by Papadopoulos *et al*. (2002) used up to 94 g/day of micro algae (*Schizochytrium sp*) containing 385 g fat/kg DM. Also, high values of VA and CLA were reported by Franklin *et al*. (1999). Most of these previous authors used micro algae species that were high in DHA and they explained the results which they depend on the effect of DHA and/ or oil on the FA's biohydrogenation in rumen. The authors speculated that the increase in VA was a result of incomplete ruminal biohydrogenation of USFA's from the fish oil but could not determine the mechanism. One possibility is that fish

oil or micro algae with high content of oil was toxic to bacteria involved in biohydrogenation. Kepler *et al*. (1966) reported that VA was a result of incomplete biohydrogenation of C18:2 fatty acid from diets containing fats. Such results were not observed by the present study due to the low content in DHA and EPA of the used micro algae which confirmed by the chemical analysis of FA in *C. pyrenoidosa* (Table 3.1.1.1), and by Ötleô and Pire (2001) who reported that *Chlorella* contains low amounts of DHA (0.22-0.30%) and EPA (0.30-0.40%).

Atherogenicity Index (AI) characterizes the atherogenicity of dietary fat; fat with higher AI value is assumed to be more detrimental to human health. In the human diet, lipids (particularly SFA) are known to contribute to coronary diseases (Williams, 2000). On the contrary, some USFA in milk has a protective effect against the risk of cardiovascular diseases, including CLA, MUFA and PUFA. In the present study, the value of AI tended to decrease but not significantly in ALG group compared to CON (Table 3.1.2.9). Moreno-Indias *et al*. (2014) reported that there was no significant difference in AI between micro algae group and control group when they gave 5 g *C. pyrenoidosa* per day in dairy goats.

3.1.1.5 Conclusion

Chlorella pyrenoidosa supplementation leads to an improvement in antioxidant enzyme activities in blood plasma but not in milk of dairy goats. From our results, we can state that the addition of 10 g C. *pyrenoidosa*/kg into the ration of dairy goats, over a period of 4 weeks, resulted in an significant increase of GR, SOD and protein of blood plasma while caused a significant decrease of GSH-Px in milk. The addition of 10 g *C. pyrenoidosa*/kg did not show any difference in milk yield or milk fat content. In addition, there was a trend to increase LCFA concentration and decrease SCFA concentration, an accumulation of C18:1 trans and decrease in *Δ9*-desaturase activity in milk. In rumen, an increase of accumulation of C18:0 and a decrease in the C18 intermediates, ($P<0.05$) MUFA concentration and ($P<0.05$) C14:1/C14:0 ratio were observed in ALG group. Therefore, our results are supportive of an antioxidant role of *C. pyrenoidosa,* indicating that *C. pyrenoidosa* is an important wholefood supplement that should be included as a key component of a diet for goats.

Experiment 2

3.2 Second experiment: Chlorella vulgaris as a dietary supplement in dairy goats.

3.2.1 Effects of dietary *Chlorella vulgaris* supplementation on enzymes activities and FA profile in rumen, blood plasma, milk, rumen enzymes and microbes of dairy goats

Abstract

Sixteen dairy goats were allocated into two groups (CON and ALG) of eight goats each, for 4 weeks to examine the effects of dietary micro algae *C.vulgaris* on animal performance, enzymes activities and FA profile in rumen, blood plasma, milk, rumen enzymes and microbes. CON served as control and received a diet without *C.vulgaris*, whereas in the ALG group, 10 g *C.vulgaris*/kg were incorporated into concentrates. Milk samples were taken weekly for chemical analysis. Blood and milk samples were also taken every two weeks to determine the activities of CAT, LPO, GST, GSH-Px, GR and SOD enzymes and FA composition. On the last day of the experiment, rumen samples were collected by a stomach tube, 3 hrs after morning feeding and frozen for rumen enzymes and q-PCR analysis. The addition of *C.vulgaris* in ALG did not affect milk yield, milk fat, milk protein and lactose content. The activities of GST, GR, GSH-Px and SOD in blood plasma were significantly higher ($P<0.05$) compared with those of the CON group. In rumen FA of ALG group, there was an increase in accumulate of incomplete biohydrogenation of C18:1 trans and C18:3n-3 and a strong accumulation of VA, C18:1 trans-10 and C18:2n-6 concentrations. The linolenic acid (C18:3n-3) concentration in the rumen leads to the formation of VA and CLA concentrations. In addition, there was an increase ($P<0.01$) in protein of blood plasma. There was no significant difference in antioxidant enzyme activity in milk ALG compared with that of CON while there was increase ($P<0.001$) in LPO in ALG compared with that of CON. In milk of ALG group, there were significantly decreased ($P<0.05$) most of the short and medium-chain FAs compared with those of CON, except for C4:0 concentration which increased ($P<0.05$). In addition, in ALG there was a significant increase in the concentration of C16:0 ($P<0.05$) in milk. Also, in milk of ALG there were a significant increase in C18:1 trans and C18:2n-6 trans concentrations while there was a significant decrease ($P<0.05$) in

C18:1 cis-9, C18:3n-3, VA, CLA and C18:1 trans-10 concentrations compared to CON during the experiment. The concentration of LCFA were significantly increased (P<0.05) in ALG compared with that of CON. In rumen fluid of ALG there was a greater protease activity (P<0.01) while lower cellulase activity (P<0.01) compared with that of CON. In addition, there was a trend to increase in ALG group in both of α-amylase and lipase without significant difference between the two groups. Comparing the population of *Fibrobacter succinogenes* and *Ruminococcus albus* to the total population of bacteria were trend to decrease in ALG as compared to CON. However, the population *Ruminococcus flavefaciens* to the total population of bacteria was a trend to increase in ALG compared to CON. In the population of *Butyrivibrio fibrosolvens* to the total population of bacteria, highly significant increase (P<0.05) were observed in the ALG compared with that of CON. Also, There was a trend to increase in Methanogens and *protozoa* in ALG compared with that of CON. It was concluded that by incorporation of very small amount of *C. vulgaris* in diet can increase the levels of protein and antioxidant enzymes in the animal plasma which may be protect the animal from the harmful effects of free radicals while the efficiency to transfer these antioxidants to milk is low. Also it can decrease the proportion of *de novo* synthesized FA. On the other hand, *Chlorella vulgaris* have not be successful to enrich milk with benefits FA such as CLA. *Chlorella vulgaris* modify the activity of rumen enzymes, total bacterial and protozoa population in rumen.

Key words: *Chlorella vulgaris*, milk, plasma, antioxidant enzymes, fatty acid profile, rumen enzymes, rumen microbes, goats

3.2.1.1 Introduction and hypothesis

The chemical composition of *C. vulgaris* compared with *C. pyrenoidosa* indicated that there is no appreciable difference between them. The protein was 57.4% vs. 67.7% and ether extracts 1.3% vs. 1.5% in *C. pyrenoidosa and C. vulgaris,* respectively. Also, there was a comparable amount in most minerals as shwenTables 3.1.1.1 and 3.2.1.1. Only the total antioxidant capacity was 3 times higher in *C. vulgaris* than *C. pyrenoidosa* (596 vs. 192 µmole of trolox/10g, respectively, Table 2.3.6.1) reported by Geetha *et al*. (2010) and Goiris *et al*. (2012).

In the first experiment the supplementation 10 g of *C. pyrenoidosa*/ kg concentrate gave marginal results not significant, but it had a significant impact on antioxidant enzymes activity in blood plasma, tendencies to modification of rumen FA profile. That means that micro algae have an effect on rumen environment (changes). So if we use another micro algae higher in total antioxidant capacity we will expect more promising significant results. For this reason we will examine deeply the effect of *C.vulgaris* which has 3 times higher antioxidant capacity (Geetha *et al.,* 2010). Due to that difference in the level of total antioxidant capacity, it was hypothesized that the addition of 10 g *C.vulgaris*/kg concentrates may be succeeded to achieve more beneficial effects. So the objective of the second experiment was investigated the effects of supplementation of dairy goats ration with *C. vulgaris* as a source of natural antioxidant on milk yield, milk composition, enzymes activities and FA profile in rumen, blood plasma, milk, rumen enzymes and microbes.

3.2.1.2 Materials and Methods

Animals

Sixteen dairy crossbred Alpine goats were allocated into two groups (CON=control and ALG=treated) of eight goats each for 4 weeks. The goats of both groups had similar daily milk productivity (as fat corrected milk 4%) and average body weight (CON 44.0 kg; ALG 43.3 kg).

Diets

Goats in CON served as controls and received a diet without micro algae *C. vulgaris* whereas in the ALG group, 10 g of *C. vulgaris* /kg were incorporated in the concentrates. Goat's nutrition was based on Onobrychis viciifolia hay and alfalfa hay as a roughage source, and a concentrate according to their requirements. The chemical composition of Onobrychis viciifolia hay, alfalfa hay, concentrate mixture, *C. vulgaris* and their minerals and FA content is shown in Table 3.2.1.1. All goats were fed individually, twice a day, in two equal meals immediately after milking, while they had free access to water. No refusals were left after each feeding. Onobrychis viciifolia hay, alfalfa hay and concentrates samples were analyzed for dry matter (DM), ash, crude protein (CP) and ether extracts (EE) according to the Weende procedure (Association of Official Analytical Chemists International, 1990) and for neutral detergent fiber (NDF) and acid detergent fiber (ADF) according to Goering and Van soest (1970). Trace elements were determined as method mentioned in the first experiment (3.1.1) by Al-Waeli *et al*. (2012). The total feed, minerals and FA intake of both groups are presented in Table 3.2.1.2.

Table 3.2.1.1 Chemical composition (in g/Kg DM) of Onobrychis viciifolia hay, alfalfa hay, concentrate mixture, *C. vulgaris* and their minerals (in mg/kg DM) and fatty acid profile (g/100 g FA).

Items	**Onobrychis hay**	**Alfalfa hay**	**Concentrate mixture**		***C. vulgaris***
			CON	**ALG**	
Chemical composition, g/Kg DM					
DM	918	939	915	915	927
OM	854	843	820	820	848
CP	134.5	188.5	129.5	129.5	677
EE	16.9	8.4	35.8	35.8	10.5
NDF	537.3	459.8	526	526	42
ADF	489.4	357	100	100	128
Minerals, mg/kg DM					
Se	0.059	0.098	0.199	0.230	0.349
Zn	11.36	11.97	139.01	123.59	15.28
Cu	5.19	6.05	10.81	10.37	2.53
Fe	237	295	326	309	1020
Mg	2417	2976	2681	2619	4548
Mn	10.20	10.49	148.10	152.11	74.67
Mo	3.021	2.283	0.664	0.651	0.173
As	0.072	0.085	0.160	0.117	1.022
Co	0.211	0.272	0.985	1.017	0.851
Ni	2.820	3.221	2.183	2.088	2.353
Sb	0.092	0.060	0.256	0.358	1.228
Pb	ND	0.203	0.501	ND	1.386
Fatty acid composition (g/100g FA**).**					
C14:0	1.01	2.88	0.00	0.00	0.81
C16:0	32.82	35.50	16.67	17.02	37.10
C16:1	1.24	2.03	0.00	0.25	6.29
C17:0	0.00	0.55	0.24	0.00	1.30
C18:0	5.43	7.11	3.01	2.98	1.35
C18:1 trans	5.71	0.00	0.24	0.36	0.34
C18:1 cis-9	5.71	0.99	29.51	29.11	2.43
C18:2 trans	0.00	0.00	0.19	0.19	0.00
C18:2 cis	16.62	32.75	47.03	46.79	22.04
C18:3n-6	0.00	0.00	0.00	0.19	0.00
C18:3n-3	2.86	1.15	0.48	0.48	22.10
C20:5n-3(EPA)	28.60	17.04	2.12	2.14	0.00
C20:2	0.00	0.00	0.00	0.00	0.00
C22:2	0.00	0.00	0.00	0.00	0.00
C22:6n-3(DHA)	0.00	0.00	0.00	0.00	0.00
C24:0	0.00	0.00	0.51	0.49	0.00

Table 3.2.1.2 Dry matter, nutrients, minerals and FA intake of experimental diets in both groups (CON and ALG).

Items	Group	
	CON	**ALG**
Total daily intake (g /head/day)		
DM	2065	2044
CP	311	316
EE	54	52
NDF	1173	1152
ADF	692	692
Minerals daily intake (mg /head/day)		
Se	0.8	0.8
Zn	353.4	321.9
Cu	48.0	47.3
Fe	1865.3	1840.4
Mg	17573	17555
Mn	367.4	378.6
Mo	13.0	13.0
As	0.7	0.6
Co	3.2	3.3
Ni	17.9	17.8
Sb	0.9	1.1
Pb	1.5	0.4
Total fatty acid intake (g /head/day**)**		
C14:0	0.22	0.23
C16:0	11.18	11.41
C16:1	0.24	0.32
C17:0	0.09	0.01
C18:0	1.96	1.97
C18:1 trans	0.98	1.00
C18:1 cis-9	10.15	10.20
C18:2 trans	0.06	0.06
C18:2 cis	18.04	18.33
C18:3n-6	0.00	0.06
C18:3n-3	0.63	0.63
C20:5n-3(EPA)	5.57	5.54
C20:2	0.00	0.00
C24:0	0.16	0.16

Milk and plasma samples analysis

For milk chemical composition, enzymes activity and FA profile in rumen, blood plasma and milk, the same time sampling collection was applied as mentioned in the first experiment (3.1.1).

Enzymes activities determination

The activities of CAT, LPO, GST, GSH-Px, GR and SOD enzymes, and protein concentration were determined in blood plasma and milk by spectrophotometer with the methods described in the first experiment (3.1.1).

Preparation and analysis of samples for Fatty acids

Fatty acids methyl ester from samples of diets, *Chlorella*, rumen, blood plasma and milk samples were determined as mentioned in the first experiment (3.1.1).

Rumen samples analysis

The last day of the experiment, rumen samples were collected by a stomach tube with a vacuum pump, 3 hrs after morning feeding. Immediately after collection, one tube (approximately 30 ml) of rumen fluid was squeezed through four layers of cheesecloth and centrifuged at 13000xg for 2 min. The supernatant was carefully decanted into two clean containers and frozen at -80^0 C for FA analysis and rumen enzymes. Another tube (approximately 30 ml) of rumen fluid was kept (without squeezing through the layers of cheesecloth) frozen at -80^0 C for q-PCR analysis.

Rumen samples preparation

The FA profile of rumen fluid was determined by the method described by O'Fallon *et al*. (2007) as mentioned in the first experiment (3.1.1).

Rumen enzymes activities

The rumen enzymes activities were determined by a colorimetric method. The assayed enzymes were 1) α-amylase (CAT; EC 1.11.1.6) assayed by the method of Bernfeld (1955). The method is based on the determination of quantity of reducing sugar (maltose) produced through the action of the enzyme with a corn starch substrate.; 2) Protease

according to the method of Folin and Ciocalteu (1929) and Anson (1938). One unit was hydrolyzed casein to produce color equivalent to 1.0 µmole of tyrosine per min at pH 7.5 at 37 ºC. Color per Folin and Ciocalteu's.; 3) Cellulase according to method described by Worthington (1988). One unit liberated 1.0 µmole of glucose from cellulose in an hour at pH 5.0 at 37 ºC.; And 4) Lipase activity was measured spectrophotometrically. The reaction was used 420µM p-nitrophenyl laurate as a substrate and 0.1 M Tris–Cl buffer (PH 8.2). The enzyme reaction was performed for 10 min at 25 ºC. The amount of p-nitrophenol liberated during the reaction was measured by absorbance at 410 nm. One lipase unit was defined as the amount of enzyme liberating 1 µmol of p-nitrophenol per minute (Praphan and Kirk, 2001).

DNA extraction protocol from rumen digesta

The protocol for isolating genomic DNA from rumen digesta described by Sharma *et al*. (2003). Two grams of rumen digesta were processed according to the following steps:

1. Weigh out 2 g rumen digesta. Grind the sample to a fine powder using liquid nitrogen.; 2. Transfer the ground sample to a 50-mL Falcon tube and add 10 mL CTAB extraction buffer [2% (w/v) CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (v/v) 2-mercaptoethanol and 100 µg/mL (v/v) proteinase K]. Note that 2-mercaptoethanol and proteinase K are added to the buffer after prewarming it to 65°C. Incubate at 65°C for 2 h with occasional mixing.; 3. Cool the solution to room temperature and add 30 µl RNase A (10 mg/mL) and incubate for 30–40 min at 37°C.; 4. Extract the homogenate with an equal volume of 24:1 chloroform: isoamyl alcohol. Mix gently and centrifuge at 10 000× g for 10 min at 4°C. Recover the top aqueous phase and repeat the chloroform: isoamyl alcohol extraction twice.; 5. Precipitate the DNA from the recovered aqueous phase with 0.6 volumes of isopropyl alcohol overnight. Recover the DNA by centrifugation at 7500× g for 15 min at 4°C.; 6. Resuspend the DNA pellet in 800 µl AP1 buffer and RNase A from the DNeasy Plant kit. Incubate the sample for 10 min at 55°C, coupled with gentle mixing 2–3 times.; 7. Add 260 µl AP2 buffer and incubate on ice for 15 min. Spin the solution, discard the pellet (if any), and either store the supernatant at 4°C or proceed to Step 8.; 8. To a 200- µl aliquot of the supernatant, add 100 µl AP3 buffer and 200 µl ethanol and mix by inverting a few times.

Apply 500 µl lysate obtained onto a DNeasy mini spin column (6000× g for 1 min). Discard flow-through and wash the column with 500 µl supplied AW buffer (three times). Leave the buffer for up to 5 min before spinning. Elute the DNA with pre-warmed (65°C) AE buffer (50 µl) twice. Note that if higher amounts of DNA are desired, total lysate obtained (1060 µl) from Step 7 can be processed. ; 9. Estimate the DNA concentration and use the required amounts for downstream processing (e.g., PCR, restriction digestion, etc.).

The developed procedure combines cell lysis with protein precipitation using CTAB, and the DNA thus obtained is precipitated following organic extractions. The precipitated DNA was solubilized in AP1 (provided with the DNeasy Plant kit) and further processed using the DNeasy Plant kit with several modifications, as indicated in pervious steps.

Concentration and purity of nucleic acids

Determination of concentration and purity of nucleic acids in the final aqueous solution was performed using spectrophotometer nanodrop. Analyzing 1 µl sample into the spectrometer nanodrop obtained the following data:

1. The concentration of nucleic acid (DNA).; 2. The purity of the sample to the proteins (index OD260/280).; 3. The purity of the sample relative to other substances such as EDTA, carbohydrates, phenols, etc. (index OD260/230).

To determine the concentration and purity of nucleic acids in aqueous solution initially transferred 1 µl ddH_2O socket device for zeroing the instrument (blank measurement). Then 1 µl of sample is transferred to the reception device and the optical density of the sample at three wavelengths: 230, 260 and 280 nm. Then the photometer showed the concentration of DNA (ng / µl) and ratios OD260/OD280 OD260/OD230. When the pointer OD 260/280 ranges between 1.8-2.0, and the OD 260/230 ratio ranges between 2.0-2.2, then the sample is regarded as being of high purity. Then, the samples were analyzed by the method of quantitative polymerase chain reaction (quantitative polymerase chain reaction q-PCR). The ability to monitor the progress of the reaction is given by measuring the level of fluorescence of the dye SYBR Green, which has the ability to

fluoresce upon binding to double-stranded molecules of DNA (dsDNA). In this way, measurements are made of the fluorescence at the end of each reaction cycle using robotic scanner. All samples were diluted so that the final concentration for q-PCR were 40 ng /microliter. Table (3.2.1.3) shows the microorganisms selected in this work as well as the respective primers for each one of them. Specifically, the PCR reaction was performed as follows: Q-PCR reaction for a final volume of 10 μl were mixed with 5 μl 2X Fast Start SYBR-GREEN Master ROX (Roche) plus 1 μl sample (concentration 40 ng / μl) and 4 μl mixture (Forward + Reverse) of the corresponding pair of primers for each organism (0.5 μM).

During the phase of the denaturation of double-stranded DNA, SYBR Green is the free form and the fluorescence produced is minimal. During the phase of the binding of the primers and the elongation, where double-stranded DNA, SYBR Green dye binds to the DNA double helix and the fluorescence produced is significantly increased (up to 1000 times). The increase of the fluorescence signal depends on the initial amount of the matrix of DNA present in the starting of PCR. The detection of the fluorescence generated from a specific place photometer detects the fluorescence produced at 520 nm. Noted that SYBR Green dye generates fluorescence when bound to any duplex DNA, such as dimers of primers or unwanted reaction products of PCR The conditions for carrying out the polymerase chain reaction was depending from the size of the replicon, which was amplified.

For replicon from 100-200 base pair (bp) was used the following protocol: Step 1: Initial denaturation at 94 ºC for 4 min.; Step 2: denaturation at 94 º C for 1 min.; Step 3: Hybridization of primers at 58 ºC for 1 min.; Repeat steps 2-4 for 41 cycles.

For replicon between 300-600 bp was used the following protocol: Step 1: Initial denaturation at 94 ºC for 3 min.; Step 2: denaturation at 94 ºC for 30 seconds. ; Step 3:

Hybridization of primers at 63 ºC for 1 min.; Step 4: Elongation at 72 ºC for 1 min.; Repeat steps 2-4 for 35 cycles.

For replicon over 1000 bp was used the following protocol: Step 1: Initial denaturation at 95 ºC for 10 min.; Step 2: denaturation at 95 ºC for 1 min.; Step 3: Hybridization of primers at 50 ºC for 1 min.; Step 4: Elongation at 72 ºC for 1 min.; Repeat steps 2-4 for 46 cycles.

For the performance of q-PCR used the device Mx3005P (Stratagene), and the corresponding software program MxPro-3005P. The results obtained by the above steps, expressed as Ct, i.e. as the cycle at which the detected respectively selected genes. Then using the software MxPro-3005P LinReg PCR and the PCR efficiency was calculated for each pair of primer. Relative quantification of each microorganism was by the following formula: (PCR efficiency) $^{-\,(\text{Ct target-Ct total bacteria})}$. This way calculated ratio of each DNA microorganism relative to the benchmark, which was the total bacterial DNA.

Table 3.2.1.3 Microbial rumen targeted and primer information.

Order Name	**Code**	**primer sequence**		**bp**
Total bacteria	BF	F	5'-CGGCAACGAGCGCAACCC-3'	130
	BR	R	5-'CCATTGTAGCACGTGTGTAGCC-3'	130
Methanogen	MF	F	5'-TTCGGTGGATCDCARAGRGC-3'	145
	MR	R	5'-GBARGTCGWAWCCGTAGAATCC-3'	145
Butyrivibrio fibrisolvens	BFF	F	5'-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3'	1200
	BFR	R	5'-CGGTCTCTGTATGTTATGAGGTATTACC-3'	1200
Ruminococcus flavefaciens	RFF	F	5'-CGAACGGAGATAATTTGAGTTTACTTAGG-3'	132
	RFR	R	5'-CGGTCTCTGTATGTTATGAGGTATTACC-3'	132
Fibrobacter succinogenes	FSF	F	5′-GGT ATG GGA TGA GCT TGC-3′	121
	FSR	R	5′-GCC TGC CCC TGA ACT ATC-3′	121
Protozoa	PF	F	5'-GCTTTCGWTGGTAGTGTATT-3'	321
	PR	R	5'-CTTGCCCTCYAATCGTWCT-3'	321
Ruminococcus albus	RAF	F	5'-CGGCAACGAGCGCAACCC-3'	175
	RAR	R	5'-CCATTGTAGCACGTGTGTAGCC-3'	175
Total fungi	FF	F	5'-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3'	120
	FR	R	5'-CAAATTCACAAAGGGTAGGATGATT-3'	120
Clostridium sticklandii	CSF	F	5'-TATCCTAAAATTACAATAGATATT-3'	1000
	CSR	R	5'-TTAAAGAAGTTCTTTTTCAATATAT-3'	1000
Peptostreptococcus anaerobius	PAF	F	5'-CGT CTW ATT TNA TGC TTG CA-3'	943
	PAR	R	5'-AGC CCC GAA GGG AAG GTG TG-3'	943
Methanosphaera stadtmanae	MSF	F	5'-CTTAACTATAAGAATTGCTGGAG-3'	150
	MSR	R	5'-TTCGTTACTCACCGTCAAGATC-3'	150
Methanobrevibacter ruminantium	MRF	F	5’-AATATTGCAGCAGCTTACAGTGAA-3’	336
	MRR	R	5’-TGAAAATCCTCCGCAGACC-3’	336

Statistical analysis

The experimental data were subjected to statistical analysis with the aid of SPSS 17.0.2 Statistical package (SPSS Inc., Chigaco, IL, USA). The body weight, the milk chemical composition, the enzyme activity, and FA of milk and blood plasma were analyzed using a general linear model (GLM) for repeated measures analysis of variance (ANOVA) with dietary treat ments (CON,ALG) and sampling time as fixed effects and their interactions (D*T) according to the model: $Yijk = \mu + Ai + Bj + Ai*Bj + eijk$, where Yijk values of observation; μ= general mean; Ai is the effect of the diet (CON vs. ALG); Bj is the effect of sampling time; Ai*Bj is the interaction between the diet and sampling time; and eijk is the residual error. Comparison of means was performed using the Bonferonni post hoc test. A value of $P \leq 0.05$ was considered significant. To statistical analysis for rumen microbial population, we used students t-test. A value of $P \leq 0.05$ was considered significant.

3.2.1.3 Results

Diet and feed intake

Table 3.2.1.1shows the chemical composition of Onobrychis viciifolia and alfalfa hay, concentrates and *C. vulgaris* and their minerals and FA content. Table 3.2.1.2 presents data of the dry matter, nutrient, minerals and FA intake. From FA intake (g/head/ day), it was noted that the major FA intake was linoleic acid (18:2n-6) and there was comparable intake in CON compared with the ALG (18.04 vs. 18.33 respectively). The second most abundant dietary FA was oleic acid (18:1 cis-9), at 10.15 and 10.20 (g/head /day) for CON and ALG, respectively. No DHA (22:6n-3) was detected. However, similar amounts of EPA (20:5n-3) were found in both groups.

Table 3.2.1.4 presents the effect of experimental treatment on body weight, milk yield and milk composition. It was noted that there was no significant difference between groups in body weight. Also, the supplement of 10 g *C. vulgaris* / kg of concentrate had no significant effect ($P>0.05$) on milk yield, milk fat percentage, milk fat yield, milk protein percentage, milk protein yield, milk lactose percentage and milk lactose yield (Table 3.2.1.4) between the two groups during the whole experimental period. On the other hand, there were significant decrease milk yield, fat corrected milk yield ($P<0.05$), fat percentage ($P<0.01$), lactose percentage ($P<0.001$), fat yield ($P<0.05$) affected by sampling time throughout the experiment.

Effects of dietary *Chlorella vulgaris* supplementation on blood plasma and milk enzymes activities

In the current work, we determined the activities of antioxidant enzymes like SOD, CAT, GSH-Px and related enzymes GR, GST, LPO in blood plasma and milk of dairy goats which received *C. vulgaris* as a source of natural antioxidant.

Blood plasma enzymes activities

The statistical evaluation of the goat's blood plasma enzymes activity is presented in units per ml of plasma and in units per mg of protein (Table 3.2.1.5). First, as units per ml plasma: the GST, GR, GSH-Px and SOD activities were significantly higher ($P<0.001$, $P<0.05$,

P<0.05 and P<0.01, respectively) in ALG compared with those of CON group. In addition, the sampling time significantly affected on the GST, GR and GSH-Px (P< 0.01, P< 0.05 and P< 0.01, respectively). Likewise, the interaction between diets and sampling time affected significantly both GSH-Px and SOD (P<0.001and P<0.01, respectively) during the experiment. The blood plasma protein concentration was significantly higher (P< 0.01) in ALG compared with that of CON group. Second, as units per mg of protein: the activities of GST, GR and SOD did not show any significant difference (P>0.05) between the two groups, while the GSH-Px was significantly higher (P<0.01) in CON compared with that of ALG during the experiment (Table 3.2.1.5).

Table 3.2.1.4 The effect of experimental treatment on body weight, milk yield and composition in both groups (CON and ALG).

Item	Diets[1]			Sampling time						Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 7 (n=16)	Day 14 (n=16)	Day 21 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
Body weight, kg	45.23	44.43	4.64	43.67^{a}	44.58^{ab}	44.83^{b}	45.08^{b}	46.00^{c}	0.137	NS	***	NS
Milk yield, g/d	1817	1727	222	1785^{ab}	1800^{ab}	1829^{a}	1733^{ab}	1712^{b}	52	NS	*	NS
Fat corrected milk yield4%, g/d	1626	1606	166	1672^{ab}	1628^{ab}	1646^{a}	1598^{ab}	1535^{b}	63	NS	*	NS
Chemical composition												
Fat (%)	3.45	3.57	0.278	3.82^{a}	3.43^{ab}	3.39^{b}	3.54^{ab}	3.36^{b}	0.099	NS	**	NS
Protein (%)	2.84	2.80	0.90	2.64^{a}	2.90^{bc}	2.85^{bc}	2.90^{b}	2.80^{c}	0.032	NS	***	NS
Lactose (%)	4.62	4.66	0.071	4.81^{a}	4.54^{b}	4.56^{b}	4.58^{b}	4.70^{a}	0.039	NS	***	NS
Fat, g/d	60.82	61.57	6.56	67.54^{a}	60.55^{ab}	60.98^{ab}	60.27^{ab}	56.64^{b}	2.65	NS	*	NS
Protein, g/d	50.92	48.22	5.29	47.04^{ab}	51.84^{a}	51.60^{a}	49.79^{ab}	47.59^{b}	1.51	NS	**	NS
Lactose, g/d	83.93	80.19	10.09	85.55	81.60	83.14	79.60	80.48	2.49	NS	NS	NS

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. vulgaris/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

Values are reduced model Bonferroni.

$^{a–d}$ Within a row, different superscripts indicate significant differences among sampling time ($P < 0.05$).

*** $p \leq 0.001$; ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$; and NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Table 3.2.1.5 Goats blood plasma enzymes activity (in Units/ ml, Units/ mg protein) and protein concentration (in mg/ml) in both groups (CON and ALG).

Item	**Diets[1]**			**Sampling time**				**Significance**		
	CON (n=8)	**ALG (n=8)**	**SEM[2]**	**Day 1 (n=16)**	**Day 14 (n=16)**	**Day 28 (n=16)**	**SEM[2]**	**D[3]**	**T[4]**	**D*T[5]**
GST U/ ml	0.161^{A}	0.249^{B}	0.019	0.152^{a}	0.213^{b}	0.249^{b}	0.020	***	**	NS
GST U/ mg protein	0.022	0.027	0.003	0.020	0.025	0.028	0.004	NS	NS	NS
GR U/ ml	0.053^{A}	0.062^{B}	0.003	0.055^{a}	0.057^{ab}	0.061^{b}	0.003	*	*	NS
GR U/ mg protein	0.007	0.007	0.000	0.007	0.007	0.007	0.001	NS	NS	NS
GSH-Px U/ ml	0.343^{A}	0.352^{B}	0.003	0.341^{a}	0.345^{ab}	0.357^{b}	0.004	*	**	***
GSH-Px U/ mg protein	0.047^{A}	0.038^{B}	0.002	0.045	0.041	0.041	0.004	**	NS	NS
SOD U/ ml	13.40^{A}	17.79^{B}	1.29	14.10	16.54	16.13	1.55	**	NS	**
SOD U/ mg protein	1.82	1.98	0.256	1.82	2.03	1.86	0.248	NS	NS	*
Protein mg/ml	7.63^{A}	9.36^{B}	0.465	7.81	8.49	9.18	0.664	**	NS	NS

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. vulgaris/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

$^{A-B}$ Within a row, different superscripts indicate significant differences between diets ($P < 0.05$).

$^{a–b}$ Within a row, different superscripts indicate significant differences among sampling time ($P < 0.05$).

*** P< 0.001; ** P < 0.01; * P < 0.05; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Milk enzymes activities

Table 3.2.1.6 shows the statistical evaluation of the goat's milk enzymes activity in units per ml of milk and in units per mg of protein. First, as units per ml milk: there was no significant difference ($P>0.05$) in the activities of CAT, GR, GSH-Px and SOD of the milk between the two groups during the experimental period. On the other hand, the milk LPO activity was higher ($P<0.001$) in ALG group, compared with that of CON group. There was no significant difference ($P>0.05$) between the two groups in the milk protein concentration during the experimental period. On the other hand, the sampling time affected significantly ($P<0.001$) the CAT and LPO. Likewise, the interaction between diets and sampling time affected significantly LPO and GR ($P<0.001$ and $P<0.01$, respectively) during the experiment. Second, as units per mg of protein: there was no significant difference ($P>0.05$) between the two groups during the experimental period in milk CAT, LPO, GR, GSH-Px and SOD activities (Table 3.2.1.6). On the other hand, the sampling time affected significantly the CAT and the blood plasma protein concentration ($P<0.001$and $P<0.01$, respectively) and the interaction between diets and sampling time affected significantly GR ($P<0.05$) during the experiment.

Table 3.2.1.6 Goats milk enzymes activity (in Units/ ml, Units/ mg protein) and protein concentration (in mg/ml) in both groups (CON and ALG).

Item	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
CAT U/ ml	180.80	189.29	3.92	198.20^{a}	178.20^{b}	178.73^{b}	3.116	NS	***	NS
CAT U/ mg protein	17.54	16.77	1.480	19.73^{a}	16.16^{b}	15.56^{b}	0.708	NS	***	NS
LPO U/ ml	0.778^{A}	0.848^{B}	0.010	0.787^{a}	0.836^{b}	0.816^{b}	0.008	***	***	***
LPO U/ mg protein	0.074	0.075	0.008	0.078	0.071	0.074	0.003	NS	NS	NS
GR U/ ml	0.076	0.083	0.004	0.079	0.074	0.086	0.005	NS	NS	**
GR U/ mg protein	0.007	0.007	0.001	0.008	0.007	0.007	0.001	NS	NS	*
GSH-Px U/ ml	0.465	0.459	0.021	0.450	0.454	0.483	0.025	NS	NS	NS
GSH-Px U/ mg protein	0.045	0.041	0.005	0.045	0.042	0.042	0.002	NS	NS	NS
SOD U/ ml	59.66	66.20	0.168	64.58	61.48	62.70	5.885	NS	NS	NS
SOD U/ mg protein	5.77	5.83	0.490	6.38	5.55	5.46	0.621	NS	NS	NS
Protein mg/ml	10.77	11.46	0.897	10.14^{a}	11.38^{ab}	11.82^{b}	0.460	NS	**	NS

Diets[1]: CON= control group without micro algae; ALG=micro algae group with 10g *C. vulgaris/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

$^{A-B}$ Within a row, different superscripts indicate significant differences between diets ($P < 0.05$).

$^{a–b}$ Within a row, different superscripts indicate significant differences among sampling time ($P < 0.05$).

*** $P< 0.001$; ** $P < 0.01$; * $P < 0.05$; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Effects of dietary *Chlorella vulgaris* supplementation on rumen, blood plasma and milk fatty acids profile

Concentration of fatty acids in rumen, blood plasma and milk.

Although milk yield and milk fat content in dairy goats fed ALG was not affected, the rumen, plasma and milk FA profile in goats fed ALG was altered compared with goats fed the CON diet (Tables 3.2.1.7, 3.2.1.8, and 3.2.1.9). In rumen, the addition 10 g of *C. vulgaris* / kg concentrates significantly increased (P<0.01) C15:1 concentration, while there was a decrease (P<0.05) C17:0 concentration compared with those of the CON group. Also, there was increase (P<0.05) in C18:1 trans, (P>0.05) in C18:2n-6 trans, and (P<0.05) in C18:3n-3 concentrations, while there was a decrease in C20:0, C20:3n-3+C22:1 and 20:4 (P<0.05, P<0.01, and P<0.01, respectively) concentrations compared with those of CON. The VA concentration was significantly increased (P<0.05) and SCSFA concentration in ALG compared with those of CON group. Also, there were significant increase in concentrations of (P<0.05) MUFA and (P>0.05) PUFA and decrease (P<0.05) S/U in ALG compared with those of CON (Table 3.2.1.7).

In plasma of ALG group, significant decrease (P<0.05) in C14:0, C17:0, C20:1 concentrations were observed compared with those of CON. On the other hand there was an increase (P<0.05) in C18:0, C18:3n-6 concentrations compared with those of CON.

In milk, the incorporation of 10g *C. vulgaris* /kg into the concentrate significantly decreased concentration (P< 0.05) most of the short and medium-chain saturates compared with those of CON except the C4:0 concentration which increased (P<0.05). In addition, in milk of the ALG group there was significantly increased (P<0.01) of C16:0 concentration (Table 3.2.1.9).

Also, in milk of ALG group a significant increase C18:1 trans (P<0.05) and C18:2n-6 trans (P>0.05) concentrations was observed, while there was a significant decrease in C18:1 cis-9, C18:3n-3, VA, CLA and C18:1 trans-10 (P<0.01, P<0.01, P<0.001, P<0.05 and P<0.001 respectively) concentrations compared with those of the CON group during the experiment (Table 3.2.1.9, and 3.2.1.10). In milk of ALG group, the LCFA concentration were significantly increased (P<0.001) and there was a trend to decrease in PUFA and

MUFA concentrations without any significant difference. Also, in milk of ALG group, there was a significant decrease in CLA and VA ($P<0.05$ and $P<0.01$ respectively) concentrations compared with those of CON group. Significant increase in *C*18:1/*C*18:0 ratio was found in ALG milk compared with the CON while the *C*16:1/*C*16:0 was significantly decreased (Table 3.2.1.11).

Table 3.2.1.7 Mean (±S.E) concentration of fatty acids (% of total fatty acids) in rumen of CON and ALG groups.

	Diets[1]		
Fatty acids	**CON(n=3)**	**ALG(n=3)**	**Significance**
C4:0	1.17 ±0.130	1.24 ±0.055	NS
C6:0	0.84 ±0.055	0.81 ±0.055	NS
C8:0	0.12 ±0.016	0.23 ±0.047	NS
C10:0	0.15 ±0.015	0.29 ±0.069	NS
C11:0	0.18 ±0.092	0.35 ±0.017	NS
C12:0	5.17 ±0.531	13.19 ±3.507	NS
C14:0	0.83 ±0.117	0.93 ±0.132	NS
C14:1	1.46 ±0.295	1.34 ±0.212	NS
C15:0	1.10 ±0.226	0.82 ±0.091	NS
C15:1	0.22[a] ±0.006	0.34[b] ±0.019	**
C16:0	15.62 ±1.459	13.50 ±0.245	NS
C16:1	0.42 ±0.097	0.25 ±0.077	NS
C17:0	0.72[a] ±0.061	0.52[b] ±0.010	*
C18:0	51.15 ±1.598	38.68 ±5.659	NS
C18:1 trans	0.97[a] ±0.217	2.56[b] ±0.386	*
C18:1 cis -9	7.46 ±0.377	7.90 ±0.176	NS
C18:2n-6 trans	0.12 ±0.020	0.46 ±0.158	NS
C18:2n-6 cis (LA)	4.48 ±0.516	4.71 ±0.150	NS
C18:3n-6	0.09 ±0.001	0.29 ±0.079	NS
C20:0	0.62[a] ±0.025	0.44[b] ±0.035	*
C18:3n-3(LNA)	1.05[a] ±0.085	1.37[b] ±0.024	*
CLA	2.16 ±0.238	3.50 ±0.537	NS
C21:0	0.24 ±0.031	0.56 ±0.149	NS
C20:2	0.12 ±0.017	0.14 ±0.073	NS
C20:3n-6	0.32 ±0.033	0.49 ±0.092	NS
C20:3n-3+C22:1	0.89[a] ±0.026	0.69[b] ±0.028	**
C20:4	0.19[a] ±0.030	0.00[b] ±0.000	**
C22:2	0.26 ±0.036	0.21 ±0.018	NS
C20:5	0.12 ±0.046	0.24 ±0.018	NS
C24:0	0.46 ±0.138	0.61 ±0.012	NS
C24:1	0.08 ±0.013	0.12 ±0.069	NS
VA	1.07[a] ±0.329	3.01[b] ±0.455	*
C18:1 trans-10	0.13 ±0.006	0.24 ±0.040	NS
SCSFA	2.46[a] ±0.106	2.92[b] ±0.108	*
MCSFA	7.10 ±0.343	14.93 ±3.695	NS
LCSFA	67.85 ±0.562	53.22 ±5.608	NS
PUFA	9.80 ±0.736	12.10 ±0.768	NS
MUFA	10.54[a] ±0.267	12.39[b] ±0.544	*
S/U	3.81[a] ±0.109	2.93[b] ±0.234	*
AI	1.18 ±0.047	1.23 ±0.099	NS
C14:1/C14	1.71 ±0.134	1.43 ±0.030	NS
C16:1/C16	0.03 ±0.004	0.02 ±0.006	NS
C18:1/C18	0.02 ±0.004	0.07 ±0.019	NS
CLA/VA	3.00 ±1.585	1.17 ±0.102	NS

Diets[1]: CON= control group without micro algae; ALG= microalgae group with 10g micro algae *C.vulgaris*/kg of concentrate mixture.

Means of fatty acids with different letters (a, b) between groups CON and ALG, differ significantly ($P \leq 0.05$). *** P< 0.001; ** P < 0.01; * P < 0.05; NS: No significance.

Table 3.2.1.8 Fatty acids concentrations (% of total fatty acids) of lipids in goats blood plasma in both groups (CON and ALG).

Fatty acids	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
C14:0	0.55 ^{A}	0.43 ^{B}	0.024	0.45	0.54	0.49	0.042	**	NS	*
C14:1	0.10	0.14	0.029	0.04^{a}	0.19^{b}	0.13^{ab}	0.050	NS	*	NS
C15:0	0.57	0.58	0.022	0.52^{a}	0.64^{b}	0.58^{ab}	0.037	NS	**	NS
C15:1	0.31	0.28	0.020	0.21^{a}	0.34^{b}	0.32^{b}	0.035	NS	***	NS
C16:0	16.44	16.33	0.215	16.65	16.11	16.40	0.373	NS	NS	NS
C16:1	0.91	0.73	0.073	0.90	0.89	0.67	0.126	NS	NS	*
C17:0	1.50 ^{A}	1.32 ^{B}	0.048	1.25^{a}	1.51^{b}	1.46^{ab}	0.084	*	*	NS
C17:1	0.44	0.39	0.022	0.39	0.40	0.46	0.039	NS	NS	NS
C18:0	21.06 ^{A}	23.54 ^{B}	0.730	22.11	22.24	22.56	1.264	*	NS	NS
C18:1 trans	0.69	0.71	0.060	0.75	0.65	0.72	0.103	NS	NS	***
C18:1 cis -9	15.29	14.94	0.456	16.86^{a}	14.61^{b}	13.88^{b}	0.790	NS	**	NS
C18:2n-6 trans	0.45	0.50	0.053	0.44	0.50	0.48	0.093	NS	NS	**
C18:2n-6cis(LA)	29.63	28.41	0.759	27.79	29.39	29.87	1.315	NS	NS	NS
18:3n-6	0.32 ^{A}	0.44 ^{B}	0.038	0.36	0.38	0.40	0.066	*	NS	NS
C18:3n-3(LNA)	2.63	2.64	0.091	1.75^{a}	3.13^{b}	3.03^{b}	0.158	NS	***	NS
C20:1	0.24 ^{A}	0.07 ^{B}	0.037	0.19	0.11	0.17	0.065	**	NS	NS
C21:0	0.06	0.03	0.021	0.14^{a}	0.00^{b}	0.00^{b}	0.036	NS	***	NS
C20:2	0.38	0.39	0.041	0.35^{ac}	0.30^{ab}	0.51^{c}	0.071	NS	*	NS
C20:3n-6	0.51	0.47	0.038	0.44	0.50	0.53	0.067	NS	NS	NS
C20:3n-3+22:1	6.72	6.51	0.194	7.60^{a}	6.33^{b}	5.92^{b}	0.336	NS	***	NS
C22:2	1.18	1.10	0.046	0.83^{a}	1.23^{b}	1.37^{b}	0.080	NS	***	NS

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. vulgaris/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

Values are reduced model Bonferroni.

$^{A-B}$ Within a row, different superscripts indicate significant differences between diets ($P < 0.05$).

$^{a–c}$ Within a row, different superscripts indicate significant differences among sampling time ($P < 0.05$).

*** $P< 0.001$; ** $P < 0.01$; * $P < 0.05$; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Table 3.2.1.9 Concentration of individual fatty acids (% of total fatty acids) in milk fat of CON and ALG groups.

Fatty acids	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
C4:0	2.30^{A}	2.99^{B}	0.218	2.51	2.40	3.02	0.267	*	NS	NS
C6:0	3.30	3.43	0.274	2.93^{a}	3.28^{ab}	3.89^{b}	0.335	NS	*	NS
C8:0	3.34	3.02	0.162	3.13	3.22	3.19	0.198	NS	NS	NS
C10:0	10.65	10.06	0.539	9.56	10.69	10.82	0.660	NS	NS	NS
C12:0	4.06^{A}	3.68^{B}	0.220	3.38^{a}	4.02^{b}	4.21^{b}	0.270	*	*	NS
C14:0	9.19	9.15	0.364	8.56	9.47	9.49	0.445	NS	NS	NS
C14:1	0.35	0.30	0.030	0.31	0.30	0.36	0.037	NS	NS	NS
C15:0	0.97^{A}	0.89^{B}	0.037	0.86^{a}	0.99^{b}	0.96^{ab}	0.045	*	*	NS
C15:1	0.23^{A}	0.19^{B}	0.021	0.20	0.22	0.22	0.025	*	NS	**
C16:0	27.45^{A}	31.20^{B}	1.109	26.22	31.74^{b}	30.02^{b}	1.357	**	***	NS
C16:1	0.73^{A}	0.83^{B}	0.065	0.94^{a}	0.70^{b}	0.70^{b}	0.080	*	**	NS
C17:1	0.23	0.24	0.028	0.28	0.21	0.22	0.035	NS	NS	NS
C18:0	10.187	9.92	0.643	11.15	9.73	9.28	0.788	NS	NS	NS
C18:1 trans	0.54^{A}	0.65^{B}	0.082	0.76^{a}	0.39^{b}	0.64^{ac}	0.101	*	**	NS
C18:1 cis-9	18.92^{A}	17.09^{B}	1.057	21.20^{a}	16.33^{b}	16.48^{b}	1.294	**	***	NS
C18:2n-6 trans	0.26^{A}	0.40^{B}	0.033	0.31^{a}	0.26^{a}	0.43^{b}	0.040	**	***	**
C18:2n-6 cis(LA)	2.53	2.08	0.181	2.58	2.06	2.28	0.221	NS	NS	NS
C18:3n-6	0.21	0.26	0.030	0.23	0.20	0.28	0.037	NS	NS	NS
C20:0	0.12^{A}	0.10^{B}	0.010	0.12^{a}	0.12^{b}	0.08^{b}	0.012	**	**	*
C18:3n-3(LNA)	0.53^{A}	0.44^{B}	0.044	0.36^{a}	0.54^{b}	0.56^{b}	0.054	**	***	NS
C20:3n-3+22;1	0.21	0.19	0.029	0.23	0.18	0.20	0.036	NS	NS	NS

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. vulgaris/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

Values are reduced model Bonferroni.

$^{A-B}$ Within a row, different superscripts indicate significant differences between diets ($P < 0.05$).

$^{a–c}$ Within a row, different superscripts indicate significant differences among sampling time ($P < 0.05$).

*** $P< 0.001$; ** $P < 0.01$; * $P < 0.05$; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Table 3.2.1.10 Fatty acids groups, CLA (% of total fatty acids), S/U and AI values of milk fat of CON and ALG groups.

Fatty acids	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
SCFA	19.59	19.50	0.849	18.13 ^{a}	19.58^{ab}	20.92^{b}	1.040	NS	*	NS
MCFA	14.23	13.73	0.557	12.80 ^{a}	14.47 ^{b}	14.65 ^{b}	0.682	NS	*	NS
LCFA	37.76^{A}	41.21^{B}	0.913	37.50 ^{a}	41.59 ^{b}	39.39 ^{ab}	1.117	***	**	NS
PUFA	4.43	3.91	0.264	4.42	3.74	4.35	0.323	NS	NS	NS
MUFA	22.96	20.81	1.092	26.02 ^{a}	19.77 ^{b}	19.86 ^{b}	1.337	***	***	NS
S/U	2.69^{A}	3.11^{B}	0.136	2.32 ^{a}	3.25 ^{b}	3.13 ^{b}	0.166	***	***	NS
AI	2.58^{A}	2.99^{B}	0.155	2.18 ^{a}	3.17 ^{b}	3.01 ^{b}	0.189	***	***	NS
CLA	0.68^{A}	0.54^{B}	0.078	0.72	0.50	0.61	0.095	*	NS	NS
VA	1.81^{A}	1.40^{B}	0.157	2.32 ^{a}	1.25 ^{b}	1.25 ^{b}	0.192	***	***	NS
C18:1 trans-	1.03^{A}	0.85^{B}	0.063	1.14 ^{a}	0.85 ^{b}	0.83 ^{b}	0.078	***	***	NS

Table 3.2.1.11 Comparison of *Δ*9-desaturase activity indexes in milk fat of CON and ALG groups.

Item	Diets[1]			Sampling time				Significance		
	CON (n=8)	ALG (n=8)	SEM[2]	Day 1 (n=16)	Day 14 (n=16)	Day 28 (n=16)	SEM[2]	D[3]	T[4]	D*T[5]
C14:1/C14	0.038	0.033	0.003	0.036	0.032	0.038	0.004	NS	NS	NS
C16:1/C16	0.028^{A}	0.027^{B}	0.003	0.037 ^{a}	0.023 ^{b}	0.023 ^{b}	0.004	**	***	NS
C18:1/C18	0.054^{A}	0.067^{B}	0.008	0.070 ^{a}	0.041 ^{b}	0.070^{a}	0.010	*	**	NS
CLA/VA	0.401	0.454	0.091	0.315	0.422	0.546	0.111	NS	NS	NS

Diets[1]: CON= control group without micro algae; ALG= micro algae group with 10g *C. vulgaris/* kg of concentrate mixture.

SEM[2]: Stander error mean of difference.

Values are reduced model Bonferroni.

$^{A-B}$ Within a row, different superscripts indicate significant differences between diets ($P < 0.05$).

$^{a–c}$ Within a row, different superscripts indicate significant differences among sampling time ($P < 0.05$).

*** $P< 0.001$; ** $P < 0.01$; * $P < 0.05$; NS: No significance.

D[3]: Effect of diet. T[4]: Effect of sampling time. D*T[5]: Interaction between diet and sampling time.

Effects of dietary *Chlorella vulgaris* supplementation on rumen enzymes activities and rumen microbes' population

Rumen enzymes activities

The effect of supplemented 10g of *C. vulgaris*/ kg concentrate mixture in ALG on rumen enzymes activities compared with CON is presented in Table 3.2.1.12. A greater protease activity was found in ALG ($P<0.01$) compared with that of CON while the cellulase enzyme activity was significantly decreased ($P < 0.01$) in ALG compared with that of CON. There was a trend to increase in both of α-amylase and lipase without significant difference between the two groups. Also, we noted that, there was a trend for increase between lipase and both protease and α-amylase.

Q-PCR and rumen microbes' population

Population sizes of targeted ruminal microbes are given in figures 3.2.1.1 to 3.2.1.5. Total bacterial population was apparently more in case of ALG than in CON, but this change was not significant in most cases. This slight increase in the total bacteria might be attributed to the increased digestibility of ALG compared with CON.

Comparing the population of fibrolytic bacteria (*Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus*) to total population of bacteria in ALG and CON was shown in (Box (A) at figures 3.2.1.1 and 3.2.1.2). The *Fibrobacter succinogenes* and *Ruminococcus albus* to the total population of bacteria decreased ($P>0.05$) in ALG as compared to CON. However, *Ruminococcus flavefaciens* to population of bacteria increase ($P>0.05$) in ALG compared with that of CON group. In case of population fibrolytic bacteria *Butyrivibrio fibrosolvens* which produced C18:1 trans, a highly significant increase ($P<0.05$) to the total population of bacteria was observed in the ALG compared with that of CON group (Box (A) at Figure 3.2.1.3). Comparing the population of C*lostridium sticklandii* to the total population of bacteria was increased in ALG compared with that of CON without significant difference (Figure 3.2.1.5), while the *Peptostreptococcus anaerobius* was almost similar in both groups (Figure 3.2.1.1). The population of total methanogens and the two major orders of methanogens (*Methanosphaera stadtmanae* and *Methanobacter*

ruminantium) increase in ALG compared with that of CON without significant difference ($P>0.05$) in both of methanogens and *Methanosphaera stadtmanae* (Box (B) at Figure 3.2.1.4 and Figure 3.2.1.1) and with significant difference ($P<0.05$) in *Methanobacter ruminantium* compared with that of CON (Figure 3.2.1.2). Total protozoa were increased in ALG compared to CON without significant difference (Figure 3.2.1.2) while the total anaerobic fungi was almost similar in both groups (Figure 3.2.1.4).

Table 3.2.1.12 Rumen enzymes activity (U/ml) in both groups (CON and ALG).

Rumen Enzymes	**Diets**		
	CON(n=3)	**ALG(n=3)**	**Significance**
α-amylase	7.08	8.31	NS
Cellulase	8.11[a]	4.40[b]	**
Protease	3.04[a]	7.54[b]	**
Lipase	3.86	5.05	NS

Diets: CON= control group without micro algae; ALG=micro algae group with 10g *C. vulgaris/* kg of concentrate mixture.

Values are reduced model Bonferroni.

[a-b] Within a row, different superscripts indicate significant differences between diets ($P < 0.05$).

[***] $P < 0.001$; [**] $P < 0.01$; [*] $P < 0.05$; NS: No significance.

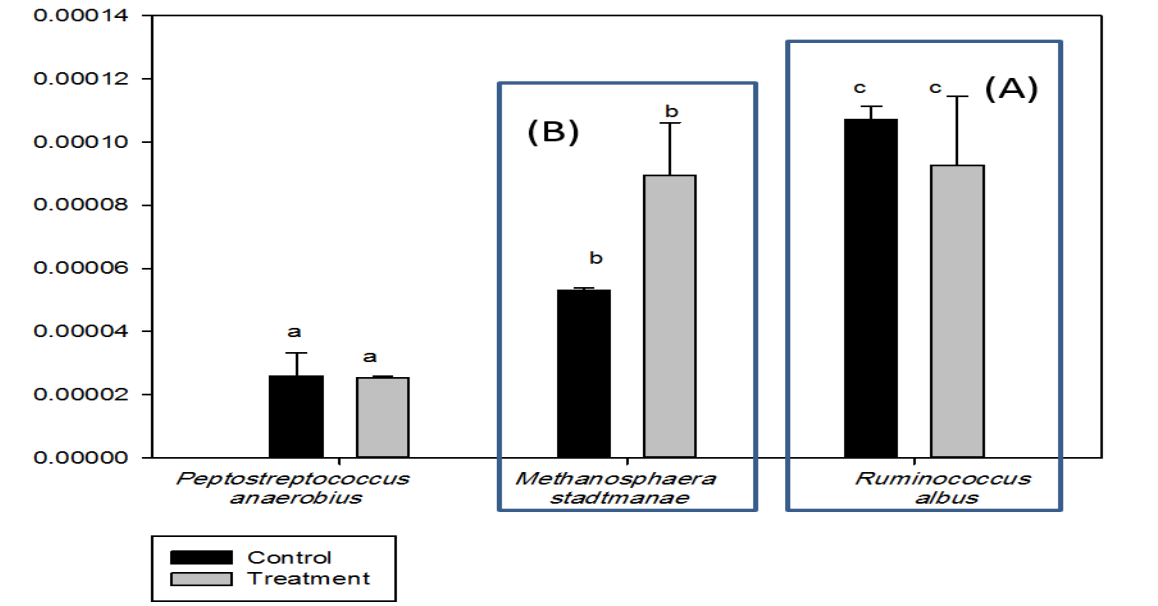

Figure 3.2.1.1 Comparing relative contribution of *Peptostreptococcus anaerobius, Methanosphaera stadtmanae*, and *Ruminococcus albus* to the total bacterial population in both groups (CON and ALG=treat.)

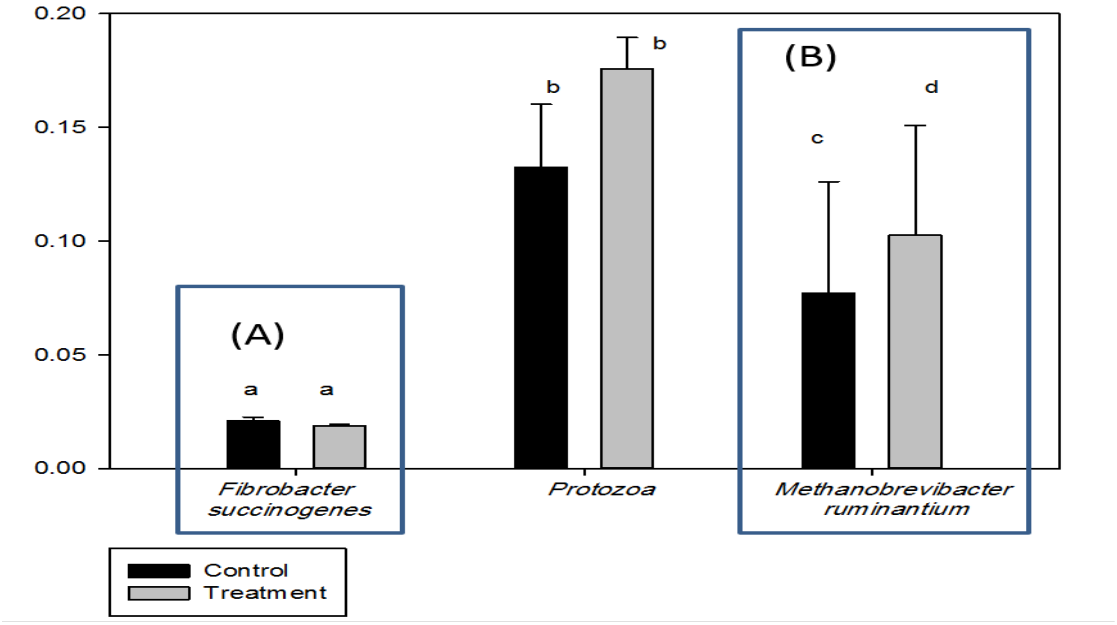

Figure 3.2.1.2 Comparing relative contribution of *Fibrobacter succinogenes*, protozoa, and *Methanobacter ruminantium* to the total amounts population both groups (CON and ALG=treat.)

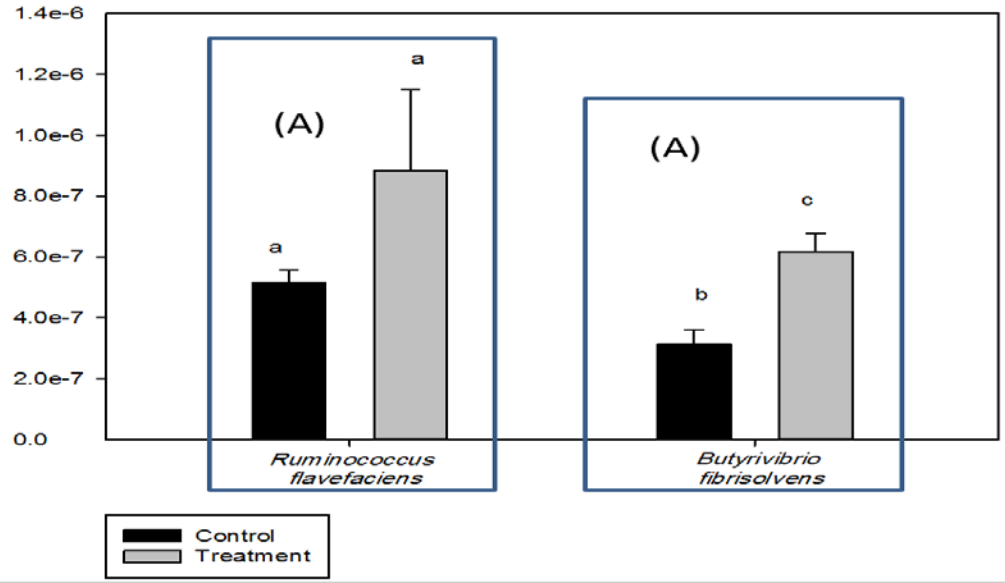

Figure 3.2.1.3 Comparing relative contribution of *Ruminococcus flavefaciens and Butyrivibrio fibrosolvens* to total bacterial population in both groups (CON and ALG=treat.)

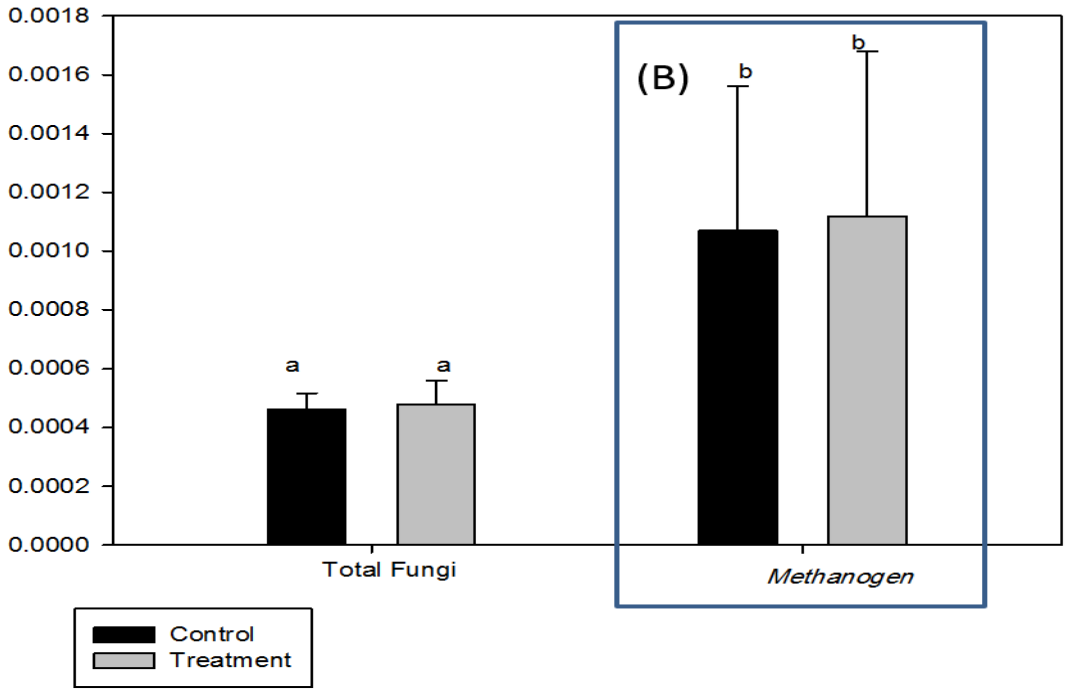

Figure 3.2.1.4 Comparing relative contribution of Fungi and *Methanogen* to the total amounts population in both groups (CON and ALG=treat.)

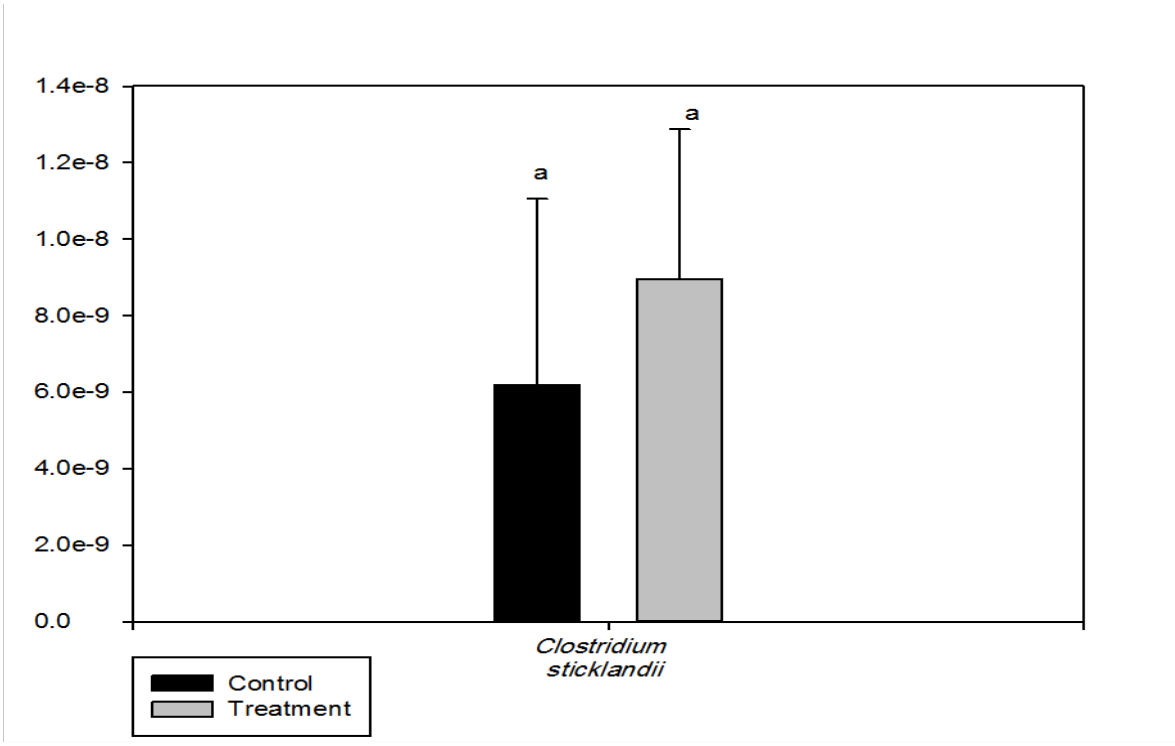

Figure 3.2.1.5 Comparing relative contribution of *Clostridium sticklandii* to the total bacterial population in both groups (CON and ALG=treat.)

3.2.1.4 Discussion

Diets and feed intake

Table 3.2.1.1 shows the chemical composition of Onobrychis viciifolia and alfalfa hay, concentrates and *C. vulgaris* and their minerals and FA content. Table 3.2.1.2 presents data of the dry matter, nutrient, minerals and FA intake. From these data, it can be seen that the intake of all nutrients was comparable between groups. In dairy cows micro algae supply reduced feed intake (Franklin *et al*., 1999; Boeckaert *et al*., 2008b).

Effects of dietary *Chlorella vulgaris* supplementation on goats performance

Table 3.2.1.4 presents the effect of experimental treatment on body weight, milk yield and milk composition. It was noted that there was no significant difference between groups in body weight. Also, the supplement of 10 g *C. vulgaris*/kg of concentrate had no significant effect ($P>0.05$) on milk yield, milk fat percentage, milk fat yield, milk protein percentage, milk protein yield, milk lactose percentage and milk lactose yield (Table 3.2.1.4) between the two groups during the whole experimental period. Comparable results in the current study were observed (Moreno-Indias *et al*., 2014) when used 5 g/day *C. pyrenoidosa* as treated supplement in dairy goats may due to the low amount of *C. vulgris* used and its low fat content (10.5 g/kg, Table 3.2.1.1).

On the other hand, there was an increase body weight ($P<0.001$), and decrease milk yield, fat corrected milk yield ($P< 0.05$), fat percentage ($P<0.01$), lactose percentage ($P<0.001$), fat yield ($P<0.05$) affected significantly by sampling time throughout the experiment. This result of increase body weight and decrease milk yield, fat corrected milk yield, daily fat, and lactose yield in both groups (CON and ALG) throughout the experimental period follows a normal trend of a lactation curve (Arguello *et al*., 2006).

Effects of dietary *Chlorella vulgaris* supplementation on blood plasma and milk enzymes activities

Blood plasma enzymes activities

The statistical evaluation of the goats blood plasma enzymes activity is presented in units per ml of plasma and in units per mg of protein (Table 3.2.1.5). There were previous studies indicating the use of micro algae as antioxidant supplement in humans (Lee. *et al*., 2010). Similar results at the blood plasma enzymes activities concerned in rats by Vijayavel *et al*. (2007) and in mice by Lee *et al*. (2003) and Mizoguchi *et al*. (2011). All these studies reported that the *C. vulgaris* can increase the antioxidant enzyme activities. A study by Travniček *et al*. (2008) in sheep reported that the supplemented with *Chlorella* increased the activity of GSH-Px in the blood of ewes and their lambs.

In the present study the SOD activity was significantly higher ($P<0.01$) in ALG compared with that of CON during the experiment. Natrah *et al*. (2007) reported a stronger antioxidant activity exhibited by methanolic micro algae crude extracts (from *e.g. Chlorella)* when compared with α-tocopherol, but lower than with synthetic antioxidant BHT. The most important substance in *Chlorella* seems to be a β-1,3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore *et al*., 2006b). Also, *Chlorella* contains carotenoids which are characterized by the ability to quench single oxygen, the inhibition of peroxide formation, and the correlation of antioxidant dependency with oxygen partial pressures. Our results are in agreement with those of Lee *et al*. (2003) who reported that *Chlorella* supplementation decreased superoxide anion production in plasma of mice and caused increasing SOD activity. The same results were confirmed in human plasma by Lee *et al*. (2010) who reported that *Chlorella* supplement exhibits antioxidant activity decreasing ROS and increasing the activity of SOD.

The GSH-Px activity was significantly higher ($P<0.05$) in ALG, compared with that of CON during the whole experimental period. Our results are in agreement with several previous studies such as Vijayavel *et al*. (2007) who reported that *C. vulgaris* inhibits production of free radicals, decreases lipoperoxidation, and increases the activity of antioxidant enzymes as SOD, CAT, GSH-Px. Lee *et al*. (2003) reported that *Chlorella*

supplementation decreases superoxide anion production in plasma of mice because increases SOD and CAT activity. The same results were confirmed in human plasma by Lee *et al*. (2010) who reported that *Chlorella* supplement exhibits antioxidant activity by decreasing ROS and increasing the activity of SOD and CAT. In addition, more studies are in agreement with our study, like Mizoguchi *et al*. (2011) who reported that *C. vulgaris* exhibits an antioxidant activity in muscle of mice, reduces the lipoperoxidation, avoiding the DNA damage.

All the previous studies explained their results depending on the biologically active compounds in *Chlorella* (Table 2.3.6.1). For instance, carotenoids have a wide pharmacological spectrum of effects. It can scavenge singlet oxygen and terminate peroxides by their redox potential because of the hydroxyl group in its structure. Carotenoids also provide protection against CCl4-caused hepatic damage by restoring the activity of hepatic enzymes like peroxidase, SOD, and CAT, which reduce ROS and lipid peroxidation (Chidambara-Murthy *et al*., 2005). Further to carotenoids and in the same context as we said, the most important substance in *Chlorella* seems to be a β-1,3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore *et al.*, 2006b).

From our results it was noted that *C. vulgaris* is able to positively affect of rations due to their original chemical composition, carotenoids, vitamins A, B, C, and E and minerals (Gouveia *et al*., 2008; Lee *et al*., 2010). There is much evidence of how the vitamins modulate intracellular signals and act as antioxidants. McDonald *et al*. (2010) reported that vitamin E is the main antioxidant but the carotenoids, vitamin A and vitamin C are also involved. Vitamin C is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Also, vitamin E is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. The antioxidant enzymes metabolize oxidative toxic intermediates and require micronutrient cofactors such as Se, Fe, Co, Zn, and Mn for optimum catalytic activity. The increase of activities for SOD and GSH-Px in ALG may be related to these bioactive compounds in *C. vulgaris* as mentioned in Table 2.3.6.1.

The antioxidant system, which involves a group of vitamins and enzymes containing trace elements working in series. The initial line of defense is by the enzymes SOD, GSH-Px and CAT. Superoxide dismutase eliminates superoxide radicals formed in the cell and prevents the reaction of the radical with biological membranes or their participation in the production of more powerful radicals. Glutathione peroxidase detoxifies lipid hydroperoxides that are formed in the membrane during lipid peroxidation (McDonald *et al*., 2010).

The result in this study reported that GST activity was significantly higher ($P<0.001$) in ALG, compared with that of CON. The antioxidant defense system in micro algae includes besides carotenoids, α-tocopherol and ascorbic acid the glutathione (Rao *et al.,* 1996; Malanga *et al.,* 1997; Rijstenbil, 2002). Glutathione, an important water-soluble antioxidant, is synthesized from the amino acids glycine, glutamate, and cysteine. Glutathione directly quenches ROS such as lipid peroxides, and also plays a major role in xenobiotic metabolism. In our results the GR activity was significantly higher ($P<0.05$) in ALG compared with that of CON. From the previous study we can state that the GR activity represents more glutathione available for quenches ROS and as a cofactor for antioxidant enzymes activity (Vijayavel *et al*., 2007).

The blood plasma protein concentration was significantly higher ($P<0.01$) in ALG compared with that of CON during the experiment. The results are in agreement with those of Su-Ching *et al*. (2012) who found that in rats the group1 and group 2 fed diet containing 4 and 8 % *Chlorella* had higher albumin concentration than the control group which had no *Chlorella*. The *Chlorella* polysaccharides increases the production of nitric oxide and nitrogen dioxide in macrophages enhancing the innate immune response, mediated by Toll-like receptors (TLR-4) (Suarez *et al*., 2010).

Albumin is the most abundant fraction (52- 62 %) of the blood protein in goats (Zubcic, 2001). *Chlorella* is the only known natural substance to raise albumin levels. Serum protein, especially albumin, is extremely accurate indicator of overall health status. Low albumin levels exist at the onset and progression of virtually every non-hereditary degenerative disease process, including cancers and cardiovascular heart disease. High protein levels have been shown to profoundly extend the life span of cells *in vitro* and to

prevent the mutation of cancer lines. On the other hand, optimal protein levels indicate sound kidney and liver function and an unstressed immune system while low albumin levels are proving to be an extremely accurate predictor of morbidity and mortality "*Chlorella Pyrenoidosa* - Questions and Answers".

Milk enzymes activities

Table 3.2.1.6 shows the statistical evaluation of the goat's milk enzymes activity in units per ml of milk and in units per mg of protein. The milk LPO activity was higher ($P< 0.001$) in ALG group, compared with that of CON group. Lactoperoxidase is one of the most abundant milk enzymes in natural form, and it represents approximately 1% of the proteins in whey (Reiter, 1985). In our results lactoperoxidase activity in milk was significantly higher ($P<0.001$) in ALG compared with that of CON during the experimental period. This result of LPO may show that the goat milk of ALG group may have higher antimicrobial activity than that of milk of CON group. Trujillo *et al.* (2007) reported the LPO is a natural antimicrobial system present in milk. Our values were lower than the values in goats reported by Zapico *et al*. (1990) and Saad de Schoos *et al*. (1999) and close to the results which were reported by Eyassu *et al*.(2004). Lactoperoxidase appears to be constitutively present in milk (Marshall *et al*., 1986; Fonteh *et al*., 2002; Isobe *et al*., 2009), although LPO activity in milk was up regulated by the lactoperoxidase system inoculation into mammary gland of cows (Isobe *et al*., 2009). However, little is known about factors regulating its expression and activity (Fragoso *et al*., 2009).

In our study the range of values for LPO activity was 0.778 to 0.848 U/ml. Previous reports have indicated different levels of LPO activity in goat milk. Zapico *et al*. (1990) reported LPO activity of 1.55 U/ml in raw goat milk from Spain. Also, LPO content in caprine milk was1.73 U/ml reported by Trujillo *et al.* (2007).

Effects of dietary *Chlorella vulgaris* supplementation on rumen, blood plasma and milk fatty acids profile

Concentration of fatty acids in rumen, blood plasma and milk.

Although milk yield and milk fat content in dairy goats fed ALG was not affected, the rumen, plasma and milk FA profile in goats fed ALG was altered compared with goats fed

the CON diet (Tables 3.2.1.7, 3.2.1.8, and 3.2.1.9). In rumen, there was increase ($P<0.05$) in C18:1 trans, ($P>0.05$) in C18:2n-6 trans, and ($P<0.05$) in C18:3n-3 concentrations, while there was a decrease in C20:0, C20:3n-3+C22:1 and 20:4 concentrations ($P<0.05$, $P<0.01$, and $P<0.01$, respectively) compared with those of CON. The concentrations of VA and SCSFA were significantly increased ($P<0.05$) in ALG compared with those of CON group (Table 3.2.1.7).

An incomplete biohydrogenation of C18:1 trans and C18:3n-3 concentrations were observed, resulting in a strong accumulation of VA and C18:1 trans-10 and C18:2n-6 concentrations. Those results in agreement with Boeckaert *et al*. (2007) who used 2 % microalgae on feed intake. Griinari and Bauman (1999) reported that CLA is synthesized as an intermediate product in the rumen during the biohydrogenation of dietary linoleic acid (C18:2n-6) or in animal tissues by *Δ*9-desaturase from VA, another intermediate in ruminal biohydrogenation. Unlike linoleic acid, biohydrogenation of linolenic acid (C18:3n-3) in the rumen leads to the formation of VA, not CLA (Harfoot and Hazlewood, 1997).

Also, there was significant increase ($P<0.05$) MUFA and ($P>0.05$) PUFA concentrations and decrease ($P<0.05$) S/U ratio in ALG compared with those of CON (Table 3.2.1.7). The increase in MUFA was a consequence of the significant increase ($P<0.05$) of VA and ($P>0.05$) of C18:1 trans-10 concentration in the rumen. The difference in rumen PUFA concentration was not significant between ALG and CON because there was no increased intake of n-3 PUFA concentration in the ALG group. The supply of n-3 PUFA concentration in the diet is one of the most important ways of improving the PUFA content of ruminant (Tsiplakou *et al*., 2008). The most striking result is the significant increase of C18:1 trans and VA concentrations in rumen of goats supplemented with microalgae accompanied by a strong decrease in C18:0 concentration (51.15 vs. 38.68 on CON and ALG, respectively, Table 3.2.1.7). The increase C18:1 trans concentrations may be related to the significant increase of *Butyrivibrio fibrisolvens* which responsible for production C18:1 trans (Boeckaert *et al*., 2008b). An incomplete biohydrogenation of PUFA in the rumen is also suggested by a lack of increase in stearic acid C18:0 concentrations in supplemented groups. The accumulation of C18:1 trans and C18:2n-6 trans concentrations indicates that the algae diet affects the second and third hydrogenation step of C18:3n-3 (C18:2 trans-11, cis-15 to C18:1

trans-11 to C18:0) and the second hydrogenation step of C18:2n-6 (C18:1 trans-11 to C18:0), (Boeckaert *et al*., 2006). The significant decrease in C18:0 concentrations may be due to the fact that the *C. vulgaris* can stimulate the activity of rumen biohydrogenation and gather the final product of the biohydrogenation C18:0 FA. This effect may be related to the high content of β-carotene and α-tocopherol in *C.vulgaris* (Table 2.3.6.1) which acts as an antioxidant and increases the activity of rumen microorganism and enhanced utilization of LCFA (Krinski, 1979; Burton *et al*., 1984; Kennedy and Liebler. 1992).

In plasma of ALG group, the addition 10g of *C. vulgaris* / kg concentrates significant decrease ($P<0.05$) in C14:0, C17:0, C20:1 concentrations compared with CON. On the other hand there was an increase ($P<0.05$) in C18:0, C18:3n-6 compared with CON. This may due to the fact that forty to sixty per cent of milk fatty acids are long-chain (predominantly C18) derived from the diet and more than 90 % of these fatty acids in milk are of plasma origin, consistent with the demonstration of little fatty acid elongation in the mammary gland (Palmquist *et al*., 1969).

In milk, the incorporation of 10g *C. vulgaris* /kg into the concentrate significantly decreased ($p< 0.05$) most concentrations of the short and medium-chain FAs compared with those of CON except the C4:0 concentration which increased ($P<0.05$). The decrease in the concentrations of short- and medium-chain FA (C8:0 to C14:0) with micro algae supplementation is in agreement with others (Donovan *et al*., 2000; Boeckaert *et al*., 2008b) who reported similar reductions in synthesized *de novo* FA in cows fed oil and /or micro algae. Generally, in dairy goats the decrease in these FA is very important and it has been positively related to “goaty” flavor in goat milk.

The reduction in the concentrations of C8:0, C10:0, C12:0, C14:0, C14:1, C15:0, C15:1 FA, short and medium chain FA in goat milk of ALG group compared with CON (Table 3.2.2.9) can be seen as a positive effect, because there is enough evidence who shows a strong link between the intake of some SFA concentrations (C12:0, C14:0 and C16:0) and the incidence of cardiovascular diseases (Hu *et al*. 2001; Mensink *et al*. 2003). In addition, in milk of the ALG group there was significantly increased ($P<0.01$) concentration of C16:0

(Table 3.2.1.9). Offer *et al*. (1999) reported that the concentration of C16:0 remained similar in supplemented and unsupplemented groups.

According to our milk results, C12:0 and C15:0 concentrations were ($P<0.05$) decreased. These results disagree with those of Moreno-Indias *et al*. (2014) when used 5 g/day *C. pyrenoidosa* as treated supplement in dairy goats. Also, in milk of ALG group a significant increase C18:1 trans ($P<0.05$) and C18:2n-6 trans ($P>0.05$) concentrations was observed, while there was a significant decrease in C18:1 cis-9, C18:3n-3, VA, CLA and C18:1 trans-10 concentrations ($P<0.01$, $P<0.01$, $P<0.001$, $P<0.05$ and $P<0.001$ respectively) compared with those of the CON group during the experiment (Tables 3.2.1.9, and 3.2.1.10). The strong accumulation of C18:2n-6 trans and C18:1 trans concentrations indicates that the algae diet affects the second and third hydrogenation step of C18:3n-3 and the second hydrogenation step of C18:2n-6 (C18:1 trans-11 to C18:0), (Boeckaert *et al*., 2006). This observation explains the reduction of both C18:3n-3 ($P<0.05$) and C18:2n-6 cis concentrations in milk of ALG group (Table 3.2.1.9).

In milk of ALG group, the concentration of LCFA were significantly increased ($P< 0.001$) and there was a trend to decrease in PUFA and MUFA concentrations without any significant difference. The same results were observed by Moreno-Indias *et al*. (2014) who used *C. pyrenoidosa* in dairy goats and they reported that there was not significant difference in both PUFA and MUFA concentrations. The difference in PUFA concentration was not significant between ALG and CON because there was no increased intake of n-3 PUFA concentrations in the ALG group. Tsiplakou *et al*. (2008) reported that increasing the supply of n-3 PUFA in the diet is one of the most important ways of improving the PUFA content of ruminant milk.

From our milk results, we observed that the C18:2 cis and C18:3n-3 concentrations which are considered as sources for CLA and VA synthesis were low in ALG (2.08 and 0.44) compared with those (2.53 and 0.53) in CON (Table 3.2.1.9). This observation may be related to the significantly high activity of *Butyrivibrio fibrisolvens* in rumen fluid of ALG compared with that of CON (Figure 3.2.1.3) which is responsible for the biohydrogenation of C18:2 cis and C18:3n-3 concentrations. *Butyrivibrio fibrisolvens*, a common rumen

bacterium, is capable of hydrogenating linoleic acid to octadecenoic acid but not to stearic acid (Boeckaert *et al*., 2008c).

In the human diet, lipids (particularly saturated FA) are deemed to contribute to coronary diseases (Williams, 2000). AI characterizes the atherogenicity of dietary fat; fat with higher AI value is assumed to be more detrimental to human health. In the present study a significant effect of ALG on AI value was found. In particular, this index was significantly higher in ALG compared to CON. This obtained result of AI due to the high value of C16:0 concentrations and the depressed value of MUFA and PUFA concentrations in ALG compared with CON. Moreno-Indias *et al*. (2014) reported that there was no significant difference in AI between micro algae group and control group when used 5 g/day *C. pyrenoidosa* as treated supplement in dairy goats.

Effects of dietary *Chlorella vulgaris* supplementation on rumen enzymes activities and rumen microbes' population

The rumen microbial population consists mainly of ciliate protozoa, anaerobic bacteria, and anaerobic fungi. This mixture of organisms digests the feed that the animal consumes, and the products of microbial digestion from the majority of nutrients that sustain the ruminant animal itself (Jenkins *et al*., 2008). These microbes are categorized into several functional groups like fibrolytic, amylolytic and proteolytic and so on, which preferentially digest structural carbohydrates, non-structural carbohydrates, proteins and other nutrients, respectively.

Ruminants have an extensive array of microbial enzymes produced in the rumen, and these enzymes play an important role in the ruminant digestive process. The enzyme activities in the rumen are diverse, and include cellulases, amylases, proteases, lipase, phytases, and tannases. The variety of enzymes present in the rumen arises not only from the diversity of the microbial community but also from the multiplicity of fibrolytic enzymes produced by individual microorganisms (Doerner and White, 1990 and Yanke *et al*., 1995). The role of protozoa in biohydrogenation was previously unclear due to the presence of accompanying bacteria (Abaza *et al*., 1975; Singh and Hawke, 1979), but recent evidence suggests that protozoa do not play a direct role in the biohydrogenation of dietary PUFA

(Devillard *et al*., 2006). Therefore, the search for natural feed supplements that modify rumen biohydrogenation has been intensified.

The most extensively studied enzyme systems of the rumen are those involved with the digestion of fiber and other associated or related plant cell wall polymers. Fibrolytic activity in the rumen arises from protozoal, fungal and bacterial sources, primarily *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Forsberg and Cheng, 1992). Biohydrogenation of USFA is carried out by rumen bacteria belonging to the *Butyrivibrio* group, which includes *Butyrivibrio*, *Pseudobutyrivibrio* and *Clostridium proteoclasticum* (Harfoot and Hazelwood, 1997; Paillard *et al*., 2007). *Ruminococcus flavefaciens* is a specialist cellulolytic bacterial species characterized from the rumen (Hespell *et al*., 1997). Currently *Ruminococcus flavefaciens* is the only rumen bacterium known to produce a defined cellulosome (Jindou *et al*., 2006). The synergism imparted by the concerted action of fibrolytic enzymes that assemble as cellulosomes is usually associated with improved cellulolytic efficiency (Bayer et al., 2004).

Rumen enzymes activities

The effect of supplemented 10g of *C. vulgaris*/ kg concentrate mixture in ALG on rumen enzymes activities compared with CON is presented in (Table 3.2.1.12). A greater protease activity was found in ALG ($P<0.01$) compared with that of CON. Generally the increase of protease activity in the rumen is caused by bacterial action (Agrawal *et al*., 1991). Most of the proteolytic bacteria in the rumen are starch degrading bacteria e.g. *Ruminobacter amylophilus* (Hobson *et al*., 1968), and *Prevotella ruminicola* (Blackburn and Hobson, 1962; Wallace and Brammall, 1985).

From our results in α-amylase and protease showed a very close cooperative action between proteolytic and amylolytic activity under experimental treatment, or perhaps the presence of some species of bacteria with both activities such as *Streptococcus bovis*, *Prevotella ruminicola*, and *Ruminobacter amylophilus* (Ali and Tirta, 2001).

Also, we noted that, there was a trend for increase between lipase and both protease and α-amylase. The lipolytic bacterium needs energy and nitrogenous substrate. Protease can

provide nitrogenous components from protein substance and α-amylase can provide substance for energy expenditure of this type of bacteria. For this reason, one may be able to see the same trend to increase in ALG between lipase and both protease and amylase. This aspect appears to be a synergistic relationship between lipolytic bacteria and other protein or starch degrading bacteria e.g. *Anaerovibrio lipolytica* and *Selenomonas ruminantium* or/and *Streptococcus bovis*, and, thus supporting each other in the degradation process (Ali and Tirta, 2001).

Cellulase enzyme was significantly decreased in ALG group compared with that of CON group. This observation may be related to the decrease of *Ruminococcus albus in* ALG group noted by q-PCR in the current study (Figure 3.2.1.1). *Ruminococcus albus* is unique relative to other cellulolytic microorganisms being examined, because the production of cellulose-hydrolyzing enzymes in *Ruminococcus albus*is conditional on the provision of phenylacetic and phenylpropionic acid (Morrison and Miron, 2000). There was a reverse trend between cellulase and protease. This result is in agreement with Kamra and Pathak (1996) who reported that important cellulose degrading bacteria are protease negative e.g. *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus.*

Q-PCR and rumen microbes' population

Population sizes of targeted ruminal microbes are given in figures 3.2.1.1 to 3.2.1.5. Most bacterial population under this study was apparently higher in case of ALG than in CON, but this change was not found significant in most cases. The changes in the total microbial community structure with dietary supplementation of marine products were observed before (Boeckaert *et al*., 2007 and Kim *et al*., 2008). On the other hand, Hino *et al*. (1993) reported that the addition of β-carotene and α-tocopherol significantly increased bacterial growth and enhanced the utilization of FA's. Backer, (1994) stated that *Chlorella* contained appreciate amount of *β*-carotene and *α*-tocopherol aslo as we mentioned in Table 3.2.6.1.

Comparing the population fibrolytic bacteria (*Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus*) to the total bacteria in both group was shown in Box (A) at figures 3.2.1.1 and 3.2.1.2. The relative contribution of *Fibrobacter*

succinogenes and *Ruminococcus albus* to the total population of bacteria decreased in ALG as compared to the CON. However, the population *Ruminococcus flavefaciensto* total population of bacteria increase in ALG compared with that of CON group. The population fibrolytic bacteria *Butyrivibrio fibrosolvens,* which produced C18:1 trans a highly significant increase ($P<0.05$) was observed in the ALG compared with that of CON group (Box (A) at Figure 3.2.1.3). Several researchers have confirmed the function role of *Butyrivibrio* species in the partial or complete biohydrogenation of C18:0 fatty acids (Jenkins *et al*., 2008). Interest in research on microorganisms involved in rumen biohydrogenation of linoleic (C18:2n-6) or linolenic (C18:3n-3) acid is currently growing. The highly significant increase in the population of *Butyrivibrio fibrosolvens* to total population of bacteria in ALG confirmed by the results which obtained in milk FA in the current study, the concentration of linoleic and linolenic acid in milk was lower and the C18:1 trans was higher ($P<0.05$) in rumen and milk in ALG compared with that of CON (Tables 3.2.1.7 and 3.2.1.9). Becker (1994) stated that *Chlorella* contained appreciated amount of β-carotene and α-tocopherol and the addition of these compounds restored cellulose digestion, clearly by stimulating the growth of cellulolytic bacteria, which are expected to be primarily responsible for the *trans*-11 biohydrogenation pathway (Martin and Jenkins, 2002). As C18:0 biohydrogenation is thought to be mainly performed by strains of the *Butyrivibrio* group (Palmquist *et al*., 2005) a primer set to study bacteria within this *Butyrivibrio* group was developed. Also, in ALG significantly increased ($P<0.05$) the C18:1 trans compared to CON during the experiment in milk profile (Table 3.2.1.9). The increase in C18:1 trans production could play a protective role for bacteria. Thus reducing membrane fluidity as a defense mechanism against lipophylic or toxic stress stimuli such as high concentration of PUFA (Bessa *et al*., 2000).

Total anaerobic fungi were almost similar in both groups (Figure 3.2.1.4). Rumen fungi are also capable of biohydrogenation, but *in vitro* data suggest that the saturation events take much longer for them to accomplish compared with rumen bacteria (Nam & Garnsworthy, 2007).

Comparing the population of C*lostridium sticklandii* to the total population of bacteria was increased in ALG compared with that of CON without significant difference (Figure

3.2.1.5), while the population *Peptostreptococcus anaerobius* to the total population of bacteria was almost similar in both groups (Figure 3.2.1.1)

The population of total methanogens and the two major divisions of methanogens (*Methanosphaera stadtmanae* and *Methanobacter ruminantium*) increase in ALG compared with that of CON without significant difference ($p>0.05$) in both of methanogens and *Methanosphaera stadtmanae* (Box (B) at Figure 3.2.1.4 and Figure 3.2.1.1) and with significant difference ($P<0.05$) in *Methanobacter ruminantium* compared with that of CON (Figure3.2.1.2).

Total protozoa were increased in ALG compared to CON without significant difference (Figure 3.2.1.2). Rumen protozoa, which are principally ciliates, contribute to the microbial lipolytic activity (Latham *et al*., 1972), but their exact role in biohydrogenation is still unknown. Moreover, ciliates were consider to contain much more CLA and C18:1 trans-11than bacteria (Devillard *et al*., 2006). These results suggest protozoa and/or their associated bacteria to be involved in particular steps of the biohydrogenation process. Protozoa in the rumen also have a partially symbiotic relationship to their bacterial neighbors. Although protests in the rumens all actively ingest and feed on bacteria, the digestive waste products are released into the rumen and can be taken up by the microflora (Coleman, 1975).

From our results, it was observed that there was an increase in methanogens and *protozoa* in ALG compared with that of CON. Methanogens are known to have symbiotic relationships involving interspecies hydrogen transfer with rumen microorganisms, especially with rumen protozoa where the methanogens can be associated intracellularly and extracellularly. Common protozoa in the bovine rumen found to have such a relationship, while the methanogens most often associated with *protozoa* which are from the orders *Methanobacteriales* and *Methanomicrobiales* (Sharp *et al*., 1998).

Methanogens can be free-living but may also form symbiotic relationships with various protozoa. This relation between protozoa and methanogenic bacteria may explain the significant increase of *Methanobacter ruminantium* in ALG group. This observation suggests that the methanogens can attach themselves to protozoa for other reasons and use them possibly just as a surface. Whether or not the methanogens play any major role in the

removal of hydrogen from the protozoa must be debatable, although evidence has been produced that when the number of methanogens in a culture of *Entodinium*s pp. increases, the rate of protozoal fermentation also increases (Hino, 1993). Hydrogen and acetate production increase as does ATP yield. Protozoans also help facilitate methanogenesis by taking up oxygen which creates a more anaerobic environment which allows the anaerobic bacteria and archae to carry out methanogenesis (Williams, 1986). It is thought that they do this by removing oxygen from the liquid parts of the rumen and then moving to the reticulum of the rumen, which creates an environment in the rumen where methanogenesis can take place (Scott *et al*., 1983).

From the present results, we noted changes in the rumen microbial community. So the marine products can change the total bacterial community structure as reported by (Kim *et al*., 2008) and they have been proved to possess a protective mechanism in the rumen biohydrogenation of USFA's (Boeckaert *et al*., 2008c). Algae PUFA remain encapsulated inside the cells of microorganisms and could be protected by the cell membrane (Papadopoulos *et al*., 2002). Also, There was a relation between the decrease in population of *Ruminococcus albus* and the significant decrease of cellulase enzyme in rumen of ALG group in the current study (Figure 3.2.1.1). *Ruminococcus albus* is unique relative to other cellulolytic microorganisms being examined, because the production of cellulose-hydrolyzing enzymes in *Ruminococcus albus* is conditional on the provision of phenylacetic and phenylpropionic acid (Morrison and Miron, 2000). From a more pragmatic viewpoint, *Ruminococcus albus* is one of the three species of cellulolytic bacteria which are predominant in herbivores.

3.2.1.5 Conclusion

From the results of this study, it can be stated that the inclusion 10 g *C. vulgaris*/kg of concentrate of the ration of dairy goats over a period of 4 weeks resulted: a) a significant increase in GST, GR, GSH-Px and SOD activities, and an increase of the protein of blood plasma. It also, caused an increase in LPO activity in milk. b) An increase in accumulate of incomplete biohydrogenation of C18:1 trans and C18:3n-3 concentrations and a strong accumulation of VA and C18:1 trans-10 and C18:2n-6 concentrations in rumen. c) An

increase in incomplete biohydrogenation of C18:1 trans and C18:2n-6 concentrations in milk. This accumulation of hydrogenation intermediate is associated with a decrease in yield of FA originating from *de novo* synthesis (<C16:0). d) Stimulatation of the activity of rumen enzymes and increased growth of total bacterial and protozoa population in rumen. This slight increase in the total bacteria and protozoa might be contributed to alleviate growth inhibition by unsaturated LCFA concentration in ALG group compared with that of CON group.

It is concluded that *C. vulgaris* in diet of dairy goats can increase the levels of protein and antioxidant enzymes in the animal plasma which may protect the animal from the harmful effects of free radicals and increase LPO in milk may be protected the raw milk product against harmful bacteria. The efficiency of transfer the effect of antioxidant to milk enzymes was low. Also, it succeed to increase the levels of C18:1 trans and C18:2n-6 but the accumulation of CLA, MUFA, and PUFA concentrations was not induced because this strain of micro algae had not high content of DHA and EPA which may serve as a means of increasing milk CLA, MUFA and PUFA concentrations in milk of ruminants. Likewise, addition of *Chlorella* which is rich in β-carotene and α-tocopherol to the diets of ruminants may have beneficial effects on the growth of rumen microbes and enhanced the utilization of FA's.

Part C:
General Discussion and Conclusions

General Discussion and Conclusions

4 General Discussion and Conclusions

In this study two strains of *Chlorella* (*C. pyrenoidosa and C. vulgaris*) were examined as natural antioxidants source for dairy goats in order to study their effects on: blood plasma and milk antioxidant enzymes activities, rumen, blood plasma and milk FA profile, rumen enzymes activities and q-PCR for rumen microbial community. The chemical composition of the two strains of *Chlorella* indicated that there was no appreciable difference between them in protein 57.4% vs. 67.7% and ether extracts 1.3% vs. 1.5% *C. pyrenoidosa and C. vulgaris,* respectively. Also, there was a comparable amount in most of the minerals. However, the total antioxidant capacity was 3 times higher in *C. vulgaris* than in *C. pyrenoidosa* 596 vs. 192 µ mole of trolox/10g DM, respectively. On the other hand, the chemical composition of the basal diets was not the same in the first and the second experiments may be due to the distinction of the roughage sources.

The addition of *Chlorella* (10 g/kg concentrates) in both experiments did not affect milk yield, milk fat, milk protein and lactose content. The supplementation did not negatively affect the milk fat content which is an important parameter in cheese yield for the dairy goat's breeders. *Chlorella* supplementation in both experiments increased the levels of protein and antioxidant enzymes in blood plasma but not in milk. This may protect the animal from the harmful effects of free radicals. However, there was significant increase in LPO activity in milk goats fed with *C. vulgaris* in the second experiment which may have a role in protection of the raw milk product against harmful bacterium that is important with inadequate transport and refrigeration facilities in developing countries.

In rumen FA profile, the addition of *C. pyrenoidosa* with the concentrate resulted in an increase of accumulation of C18:0 concentration, a decrease in the C18 intermediates and C18:3n-3 concentration which leaded to decrease of VA and CLA concentrations in the first experiment while the addition of *C. vulgaris* with the concentrates in the second experiment leaded to accumulation an incomplete biohydrogenation of C18:1 trans and C18:3n-3 concentrations and resulted in a strong accumulation of VA and C18:1 trans-10 and C18:2n-6 concentrations. The accumulation of linolenic acid (C18:3n-3) concentration in the rumen leaded to increase the formation of VA and CLA in the second experiment.

Also, in both experiments, the addition of *Chlorella* decreased the yield of FA originating from *de novo* synthesized (<C16:0) in milk, increase trans C:18 FA's and decrease cis C:18 FA's in plasma and milk with the same trend. In the second experiment in ALG group the adding the *C. vulgaris* improved protease activity ($P<0.01$) and reduced cellulase activity ($P<0.01$) in rumen samples. In addition, significantly increased ($P<0.05$) *Butyrivibrio fibrosolvens*, insignificantly increased *Ruminococcus flavefaciens*, C*lostridium sticklandii*, methanogens and protozoa while insignificant reduction of *Ruminococcus albus* were observed in rumen samples of the ALG group compared with those of CON group.

Finally, from our findings, we conclude that, addition of *Chlorella* (10g / kg concentrates) can be used safely in dairy goats without any harmful effect on animal performance. *Chlorella* supplementation of this level succeed as a natural antioxidant to increase the antioxidant enzyme activity in blood plasma but not in milk. So, our study recommend to increase the amount of *Chlorella* in further studies to investigate the effect of *Chlorella* on milk antioxidant enzymes. The addition of *Chlorella* (10g/kg concentrates) decreased FA originating from *de novo* synthesized. In dairy goats the decrease in these FA is very important and it has been positively related to "goaty" flavor in goat milk. This amount of *Chlorella* increased most rumen enzymes and microbes under this study.

We also conclude that the effect of the strains of *Chlorella* may be depended on the level of total antioxidant capacity. In the present study, the *C. vulgaris* which contain 3 times higher in the total antioxidant capacity which used in the second experiment succeeded to achieve more beneficial effects on blood plasma and milk antioxidant enzymes activities; rumen, blood plasma and milk FA profile than *C. pyrenoidosa* in the first experiment. Thus, it is clear that the two strains under the same species of micro algae have a different essential role in regulation of enzyme activity and FA synthesis may be due to the level of the total antioxidant capacity and the chemical composition of basal diets. So, our study also recommends in further studies to use the microalgae which high in antioxidant to achieve benefit results.

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