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Effect of Different Levels from Linseed Oil and Linseed Oil Beads on Rumen Fermentation and Microbial Parameters Using Gas Production System and Rumen Simulation Technique

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ABSTRACT

Despite the fact that the ruminant diet is rich in Polyunsaturated Fatty Acids (PUFA), ruminant products, such as meat, milk and dairy contain mainly Saturated Fatty Acids (SFA) because of bacterial lipolysis and subsequent biohydrogenation of ingested PUFA in the rumen. The link between SFA consumption and coronary heart disease is well established. The objectives of this study in this field were to find ways of manipulating ruminal microbes to increase the flow of PUFA from the rumen into meat and milk. The main objective of the current study was to evaluate new encapsulation method using biopolymers to protect linseed oil from rumen biohydrogenation and its effect on rumen fermentation and microbial parameters. Gas Production System (GPS) and rumen simulation technique (rustic system) were used in two separated experiments. Within GPS experiment, a mixture was used as a substrate which was comprised of meadow hay and barley meal in the ratio of 60:40. Linseed beads 1 (15% oil), linseed beads 2 (20% oil) and linseed oil were supplemented to the substrate at four levels (0, 2, 4 and 6%) from substrate as DM basis. The overall results from GPS trial concluded that beads 1, beads 2 and linseed oil in the free form at different levels did not have a negative effect on fermentation pattern in most cases. The only negative effect for linseed oil treatment was on protozoa count and it concluded that the encapsulation process has a good benefit to avoid this negative effect for linseed oil. The closed level for control case from these three treatments was 4% which was used for the next experiment. On the rustic system, the basal substrates consisted of grass hay and concentrate (corn meal+rapeseed meal) in a 1:1.5 ratio and were added in the portions of 12 g DM day^{-1}. The treatments were as following: Control (Basel substrate without supplements), beads 1 (control+4% of linseed beads containing 15% oil), beads 2 (control+4% of linseed beads containing 20% oil) and linseed oil (control+4% of linseed oil). Different fermentation parameters and microbial parameters were measured. The overall conclusion was that unprotected linseed oil had a significant negative effect on ammonia nitrogen and *Holotricha* protozoa. It also indicated that unprotected linseed oil had a negative effect on total bacteria count but not significant and no significant effect on other fermentation parameters. The results obtained using rustic system as a long term of incubation confirmed the results obtained by GPS as a short term of incubation. The results obtained conclude that unprotected linseed oil supplementation has a negative effect on rumen microflora, however, this negative effect could be avoided by protect linseed oil.

Key words: Linseed oil, rumen, PUFA, rustic, GPS

INTRODUCTION

With the expectation of an increased production response, dietary fat is used for dairy cows in order to diminish the gap between Net Energy Intake (NEI) and requirements to high milk yield and maintenance (Weisbjerg *et al.*, 2013). Furthermore, uses of supplementary fat to manipulate milk Fatty Acid (FA) profiles (Larsen *et al.*, 2012) and reduce methane excretions (Beauchemin *et al.*, 2007a), has gained interest. In addition, due to the attribution of several health benefits that fatty acids bring (Park, 2009), many strategies have been used to improve the lipid profile of milk from ruminant animals. Providing sources of supplemental fat to animals has shown to be a good alternative to enrich milk fat with fatty acids important to human health, such as Conjugated Linoleic Acid (CLA) and omega-3 fatty acids (n-3) (Toral *et al.*, 2010; Whitlock *et al.*, 2006).

Dietary lipids are rapidly hydrolyzed and biohydrogenated in the rumen resulting in meat and milk characterized by a high content of saturated fatty acids and low Polyunsaturated Fatty Acids (PUFA), which contributes to increases in the risk of diseases including cardiovascular disease and cancer. There has been considerable interest in altering the fatty acid composition of ruminant products with the overall aim of improving the long-term health of consumers (Kim *et al.*, 2000). Much study has been aimed at improving the fatty acid profile of milk so that the proportion of saturated fat is decreased and that of unsaturated fatty acids is increased (Jenkins and McGuire, 2006). Recent efforts have focused on increasing the concentration of LC-PUFA, particularly that of omega-3 (n-3) FA in milk. These fatty acids have been shown to enhance infant growth and visual and cognitive development and reduce the risk of cardiovascular disease, autoimmune disorders, type 2 diabetes, hypertension, rheumatoid arthritis and certain cancers (Simopoulos *et al.*, 1999).

However, increasing dietary PUFA flow to small intestine is a major challenge in ruminant animals as dietary PUFA are extensively biohydrogenated in the rumen and only small quantities (<10%) of dietary PUFA are protected and become available for absorption in the small intestine (Wu *et al.*, 1991; Jenkins, 1993). Different methods have been used to provide protection for the PUFA against rumen biohydrogenation. Exemplary methods include mechanical and heat treatment of oil seeds, formaldehyde treatment of protein-lipid mixtures, formation of calcium salts, and linkage by amide bonds (Tymchuk *et al.*, 1998; Sinclair *et al.*, 2005). Currently, the only commercially used method to protect unsaturated fatty acids from microbial action in the rumen is by supplementation with fatty acid calcium salts. Although saturated and mono-unsaturated fatty acids react well with calcium forming an insoluble product that resist rumen degradation (biohydrogenation), PUFA do not readily react to form calcium salts and therefore the salts provides little to no protection against the breakdown of PUFA by rumen biohydrogenation (Lundy *et al.*, 2004; De Veth *et al.*, 2005; Castaneda-Gutierrez *et al.*, 2007). Therefore, there is a need for finding a more effective method by which PUFA in fat supplements can be protected to bypass the rumen to become available for absorption in ruminant animal's lower digestive tract. The main objective of the current study is to evaluate new encapsulation method using biopolymers to protect linseed oil from rumen biohydrogenation using gas production system in short term of incubation and rustic system in long term of incubation.

MATERIALS AND METHODS

Preparing linseed oil beads: Sodium alginate/k-carrageenan gel was prepared by dissolving sodium alginate/k-carrageenan (1:1 w:w) in distilled water to get a concentration of 2.5% (w/v)

alginate/carrageenan gel. The gel solution was mixed thoroughly using an overhead mechanical stirrer until complete dissolution had occurred. The linseed oil emulsion gel was prepared by mixing 15 and 20 vol% linseed oil with alginate/carrageenan gel (v/v) using Tween80 as an emulsifier (0.5 mL/100 mL gel) to get tow linseed oil beads concentration. Uniform linseed oil beads were formed through injection of the linseed emulsion gel solution using the Encapsulator instrument (model IE-50R was purchased from Encap. Biosys., Switzerland) under specific conditions as follows: Nozzle: 1 m, frequency 1700 Hz, flow rate: 4 mL min^{-1} and air pressure of 1 bar. The formed beads were received in 2.5% $CaCl_2$ (w/v) and left in the hardening solution for up to 30 min. The linseed oil beads were filtered and dried using oven dryer (45°C).

***In vitro* studies**

Gas Production System (GPS): The gas production system technique allows analyzing of gas kinetics to select the most interesting level of additives, which affect rumen fermentation and inhibit the methanogenesis. In this system of microorganisms cultivation, during the incubation, the dietary carbohydrates are transformed to the short chain fatty acids and gases, mainly CO_2 and CH_4.

Incubation and sampling procedures: Rumen fluid was collected from 3 ruminally fistulated Polish Holstein-Friesian cows (weight 680 kg). The cows were fitted with ruminal cannula by surgical procedure approved by the guidelines of Local Ethical Board for animal's treatment. The animals diet was composed with grass silage (7 kg DM), corn silage (5 kg DM), meadow hay (1.8 kg DM) and concentrate (1 kg DM). The animals were fed at the rate of 2.5 kg dry matter/100 kg of body weight. The collected rumen fluid (before morning feeding) was mixed and squeezed through a 4-layers cheesecloth into a Schott Duran® bottle (1 L) with an O_2-free headspace and immediately transported to laboratory at 39°C where was used as a source of inoculum. A mixture of meadow hay and barley meal in the ratio of 60:40 was used as a substrate. Linseed beads 1 (15% oil), linseed beads 2 (20% oil) and linseed oil were supplemented to the substrate at four levels (0, 2, 4 and 6%) from substrate as DM basis. Each level was tested in four replicates accompanied by blank vessels (no substrate). In the experiment, a 100 mL gas-tight syringes (HaberleLabortechnik, Germany) was used. According to the methodology of Navarro-Villa *et al.* (2011), a set of incubation gas-tight syringes comprised of a four gas-tight syringes for each tested level and a four control syringes without any supplements as well as 4 syringes as blanks (without substrate). The 400 mg of milled substrate (consisted of 240 mg of meadow hay and 160 mg of barley meal, both ground to 1 mm) was added to the incubation gas-tight syringes. rumen fluid was mixed with the buffer solution (292 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \times 7H_2O$, 64 mg $CaCl_2 \times 2H_2O$, 4 mg Na_2CO_3 and 600 mg cysteine hydrochloride per 1 L of double distilled water (ddH_2O) in the 1:4 (v/v) ratio. Incubations were run at 39°C under CO_2 in 40 mL buffered rumen fluid added to pre-warmed gas-tight syringes containing substrate and supplements and then the samples were incubated at 39°C for 24 h.

Determination of basic rumen fermentation parameters and methane production: After an incubation of 24 h, the pH of rumen fluid was measured (pH-meter CP-104, ELMETRON). The overall quantity of gas was determined according to Makkar and McSweeney (2005). The volume of produced gases was measured at 0.5, 1, 2, 4, 6, 12 and 24 h of incubation. Quantitative

analysis of ammonia concentration was carried out by a modified Nessler's method modified by Cieslak *et al.* (2002). Briefly, after an incubation of 24 h, 3.6 mL of buffered rumen fluid was sampled from the serum flasks and centrifugated at 12000 rpm for 5 min. A 100 μL of supernatant was then transferred to the tube contained 200 μL of 1% polyvinyl alcohol, 200 μL of 20% potassium sodium tartrate, 200 μL of Nessler reagent and 19.3 mL of ddH_2O and incubated 10 min at the room temperature. After incubation, the samples were checked spectrophotometrically. The methane concentration was checked after 6, 12 and 24 h from incubation. A 500 μL gas was sampled from the gastight syringe (GASTIGHT® Syringes, Hamilton Bonaduz AG, Switzerland) into SRI310 gas chromatograph equipped with a Thermal Conductivity Detector (TCD) and Carboxen-1000 column (mesh side 60/80, 15 FT×1,8 INS.S, SUPELCO). Short Chain Fatty Acids (SCFA) were calculated according to Getachew *et al.* (2002) equation:

$$mmolSCFA = -0.00425+0.0222 (GP)$$

where, GP is 24 h net gas production.

Determination of microbial population: For protozoa determination, the contents of the serum flasks after incubation were mixed properly and 1 mL sample was mixed with 6 mL of 4% formaldehyde and to analyze the total number of bacteria, 20 μL was added to 6980 μL of Hayem solution (2.5 g $HgCl_2$, 25 g Na_2SO_4 and 5.0 g NaCl per 1 L of double distilled water). The protozoa were counted microscopically (150x) in the drop of rumen fluid with the defined volume, with the division on the *Holotricha* and *Entodiomorpha* groups. The bacteria were obtained with Thoma chamber (0.02 mm depth, Blau Brand, Wertheim, Germany). Quantitative and qualitative changes of the total number of methanogens were determined by molecular biology techniques, including Fluorescence *In Situ* Hybridization (FISH) according to Soliva *et al.* (2004) and Pers-Kamczyc *et al.* (2011).

Rustic system

Experimental feed: Rumen Simulation Technique (RUSITEC) (Czerkawski and Breckenridge, 1977) and modified as described by Machmuller *et al.* (2002) was used. This dynamic system of rumen microorganisms cultivation is built of 8 fermenters with a capacity of 1 L each. The advantage of this system compared to the previously described method, is the long-term (10 day) incubation of medium in the experimental conditions similar to natural (with simultaneous delivery of the product and the draining of the substrate). The 4% level from linseed oil and linseed oil beads was supplemented to basal substrates compared with the unsupplemented basal substrates. The basal substrates consisted of grass hay and concentrate (corn meal+rapeseed meal) in a 1:1.5 ratio and were added in portions of 12 g DM day^{-1}. Linseed products evaluated were: Linseed beads containing 15% oil in DM, linseed beads containing 20% of oil in DM. The treatments were: Control (basal substrate without supplements), beads 1 (control+4% of linseed beads containing 15% oil), beads 2 (control+4% of linseed beads containing 20% oil) and linseed oil (control+4% of linseed oil). (In order to simulate the chewing activity of the ruminant, the grass hay was minced in a regular food mixer). Corn and rapeseed meal, which represented the concentrate, were ground to a diameter of 1 mm. Mineral-vitamin premix (Contained (per kg): Ca, 140 g, P, 70 g, Na, 80 g, Mg, 30 g, Se, 15 mg, vitamin A, 150 mg, vitamin D3, 3 mg, vitamin E, 1.67 g) was added by

0.07 g day^{-1}. The experiment in rustic lasted 10 days. To ensure a steady state within the vessels, an adjustment period for the first 6 days was allowed. Measurements were on days 7-10. The numbers of samples were duplicated from 4 experimental days (n = 8).

Rumen inoculum and artificial saliva: Vessel inoculum was obtained from three ruminally cannulated Polish Holstein-Friesian dairy cows. The fermentation inoculum (solid and liquid) was collected before morning feeding and samples from each cow were combined and thoroughly mixed to provide a single 4 L sample of rumen fluid containing about 400 g of digesta solids. The rumen fluid containing solid digesta was transported aerobically immediately to the laboratory at 39°C and transferred to a water bath with continual CO_2 purging until the RUSITEC was ready to receive them. Artificial saliva was created by dissolving 9.80 g Na_2HCO_3 and 4.68 g Na_2HPO_4/L of double distilled water (ddH_2O). To this, 10 mL L^{-1} of salt mix was added, which contained 47 g NaCl, 57 g KCl, 5.40 g $CaCl_2$ and 12.80 g $MgCl_2$ dissolved in a 1 L of ddH_2O. Additionally, 3 mL of: 0.64 g $ZnSO_4$, 0.34 g $MnSO_4$ and 0.02 g $CoSO_4$ dissolved in a 1 L of ddH_2O mixture were added.

Rustic fermenter set-up: The rustic apparatus consisted of 4 air-tight 1 L vessels immersed in a water bath maintained at 39°C. Each vessel was filled with 820 mL of strained rumen fluid, 100 mL of artificial saliva, one nylon bag (100 μm pore size) containing 11 g DM of digesta solids and another containing the experimental diet (12 g DM/bag). The incubation vessel was then sealed and saturated with N_2 to obtain anaerobic conditions. Bags were moved up and down by the motor-driven arms continuously. On the first day of each experimental run two nylon bags, one filled with solid ruminal content, the other filled with the respective dietary treatment, were put into each fermenter. After an incubation of 24 h, each vessel was opened and bags with rumen digesta solids were removed, squeezed and washed with artificial saliva. The new bags, containing the experimental diet, were inserted into the incubation vessels. On subsequent days, the bag which had been incubated for 48 h was withdrawn and replaced with a new one in a similar way. Afterwards, each nylon bag was incubated in the fermenter for 48 h. Anaerobic conditions were re-established in the fermenters by rinsing the system with gaseous N_2 for 3 min (3 L min^{-1}) after the daily supply of substrate was completed. The buffer flow rate was kept at 500 mL day^{-1}. Artificial saliva was continuously infused using a peristaltic pump adjusted to attain a liquid dilution rate of 0.035 h. Displaced effluent and fermentation gases were collected in an effluent vessel and gas collection bag respectively. To stop fermentation in the effluent vessels, 10 mL of 6 N HCl was added to each.

Sampling: The experiment ran for 10 days with sampling of rumen fluid for rumen fermentation parameters on d 7, 8, 9 and 10 at 3 h before the addition of the new feed bag. The pH was measured immediately after collection using a pH meter. Rumen fluid after incubation (3.6 mL) was immediately analyzed for NH_3-N. Volatile Fatty Acids (VFA) was determined in 3.6 mL of collected rumen fluid mixed with 0.4 mL of 46 mM $HgCl_2$. Samples for VFA were stored at -20°C prior to analysis. For estimation of microorganism populations, 1.5 mL of rumen fluid was collected. Fermentation gases were collected over 24 h in gas-tight bags (TECOBAG 81; Tessaraux Container GmbH, Burstadt, Germany) connected to the effluent vessels by flushing the RUSITEC system with gaseous N_2 before removing the bags.

Analytical methods: Samples of fermenter fluid were analyzed for pH, NH_3 and oxi-reductase potential (to monitor the anaerobic conditions) daily with the respective electrodes connected to a pH meter data-processing unit (model 713; Metrohm, Herisau, Switzerland). Substrates and substrate residues after 48 h of incubation were dried at 70°C and analyzed for the amount of DM (DM digestibility) according to AOAC (2007). The ammonia concentration was checked by the colorimetric Nessler's methods as modified by Cieslak *et al.* (2002). Fermentation gases were collected over 24 h in gas-tight bags connected to the fermenters. The fermentation gases were collected completely by flushing the Rustic system with gaseous N_2 before uncoupling the bags from the fermenters. The volume of the fermentation gases collected was quantified by pressing the gas into a closed tube filled with water and measuring the amount of water displaced. Fermentation gases were analyzed for the concentration of CH_4 by a SRI310 gas chromatograph (SRI Instruments, Torrance, CA, USA) equipped with a thermal conductivity detector and Carboxen-1000 column (mesh size 60/80; Sigma Aldrich, Poznan, Poland) according to Szumacher-Strabel *et al.* (2011). For the determination of VFA, 1.8 mL of the fermenter fluid samples was stabilized with 0.2 mL of a 46 mM-$HgCl_2$ solution and frozen until analysis by GC (GC Star 3400 CX; Varian, Sugarland, TX, USA) as outlined by Tangerman and Nagengast (1996). The population of bacteria was obtained with Thoma counting chamber (Blau Brand®, Wertheim, Germany; Ericsson *et al.*, 2000). Counts of protozoa (i.e., *Entodiniomorphs* and *Holotrichs*) were determined according to Michalowski *et al.* (1986). Quantification of methanogens was carried out with the fluorescence in situ hybridization technique described by Soliva *et al.* (2004) and Pers-Kamczyc *et al.* (2011).

Statistical analysis: The data were analyzed using general linear method of statistical analysis system (SAS., 2004). Duncan's multiple range test (Duncan, 1955) was carried out for separation among means. Data of gas production system, batch culture system, rumen simulation technique system, nutrient digestibility and milk fatty acid profile were analyzed according to one way analysis of variance where the model was:

$$Y_{ij} = \mu + T_i + E_i$$

Where:
Y = Effect of the observation
μ = Overall mean
T = Effect of the treatment
E = Experimental error

RESULTS AND DISCUSSION

Effect of different levels from linseed oil and linseed oil beads on rumen fermentation and microbial parameters using GPS: The gas production system was used to evaluate effects of the linseed oil and linseed oil beads on gas kinetics and some other rumen fermentation and microbial parameters, also, to determine the best ratio can be used for subsequent investigations. The results of the effect of different supplementation levels from linseed oil, linseed oil beads 1 and linseed oil beads 2 are illustrated on Table 1, 2 and 3, respectively. The levels were, control (without supplementing), 2, 4 and 6% (on DM basis) from linseed oil, beads 1 and beads 2.

Table 1: Effect of different levels from linseed oil on different rumen fermentation and microbial parameters using GPS

	Addition of linseed oil (%DM)				
Item	Control	2	4	6	SEM
pH	6.14	6.12	6.13	6.08	0.02
N-NH_3 (mg dL^{-1})	12.88^{b}	13.19^{ab}	13.34^{ab}	13.66^{a}	0.09
SCFA	1.69	1.73	1.76	1.76	0.01
TGP 0.5 h	4.00	3.50	4.00	3.25	0.15
TGP 1 h	5.50	5.25	5.00	5.00	0.16
TGP 2 h	8.75	8.50	8.25	8.75	0.20
TGP 4 h	18.25	17.75	18.00	18.00	0.22
TGP 6 h	27.25	26.50	26.75	26.25	0.27
TGP 12 h	51.25	51.50	52.00	52.00	0.41
TGP 24 h	76.50	78.25	79.25	79.50	0.63
CH_4/6 h	3.50^{a}	3.11^{ab}	3.01^{ab}	2.83^{b}	0.09
CH_4/12 h	7.31	7.04	7.35	6.70	0.16
CH_4/24 h	12.26	11.98	12.14	11.95	0.20
B.10^{7} mL^{-1}	18.81	19.03	19.25	19.25	0.36
Meth. B. 10^{7} mL^{-1}	0.49^{a}	0.42^{b}	0.46^{a}	0.43^{ab}	0.01
Ento. 10^{3} mL^{-1}	35875.00^{a}	32112.50^{ab}	22925.00^{b}	29837.50^{ab}	1317.55
Holo.10^{3} mL^{-1}	5888.75^{a}	6125.00^{a}	5206.25^{b}	5162.50^{b}	120.01

^{a,b}Different superscripts indicate statistical significance at the same row ($p<0.01$), TGP: Total gas production, after (0.5, 1, 2, 4, 6, 12 and 24 h), methane concentration (CH_4) after (6, 12 and 24 h), pH, SCFA: Short Chain Fatty Acid, ammonia concentration (NH_4), B: Rumen bacteria, Holo: *Holotricha* protozoa, Ento: Entodonerma protozoa and methanogens bacteria

Results of Table 1 indicated non significantly ($p>0.01$) lower ruminal pH values with the different levels of unprotected linseed oil than control. The different levels of beads 1 showed significantly lower ruminal pH than control (Table 2). Also, lower pH values were recorded for the different levels of beads 2 with significant for the 6% level compared to control (Table 3). The pH results of linseed oil supplementation at the different levels are in agreement with previous *in vivo* studies using different lipid sources, including fish and sunflower oils (Fievez *et al*., 2003; Beauchemin *et al*., 2007b; Toral *et al*., 2009). However, Shingfield *et al*. (2003) reported a higher pH when fish oil was included in the diet of cows, which was attributed to associated decreases in DM intake. Also, studies in sheep have reported a numerically higher rumen pH when fish oil was included (Wachira *et al*., 2000). The lack of a difference in vessel pH within beads 1 and beads 2 treatments is consistent with other studies that have investigated the effect of seed associated on pH *in vitro* (Wang *et al*., 2002). However, studies in sheep and cattle have reported a similar ruminal pH when supplemented with whole linseed compared with a protected source of palm oil (Wachira *et al*., 2000; Scollan *et al*., 2001). Generally, in spite the lowering of pH values at different levels than control within all treatments (Table 3-5), the mean of pH values remained always within a physiological range (normal range of pH between 6.0 and 6.7) reported by Buccioni *et al*. (2012).

Results of unprotected linseed oil (Table 3) indicated non significant ($p>0.01$) higher ammonia for 2 and 4% levels than control, while, 6% level has significant ($p<0.01$) higher ammonia than control. However, non significant lower ammonia values were detected for the different levels of beads 1 (Table 4) and beads 2 (Table 5) than control. Ruminal ammonia concentrations must be always greater than the 100 mg L^{-1} reported by Van Soest (1994) as optimal for the efficiency of

Table 2: Effect of different levels from linseed oil beads 1 (15% oil) on different rumen fermentation and microbial parameters* using GPS

	Addition of linseed beads1 levels (%DM)				
Item	Control	2	4	6	SEM
pH	6.31^{a}	6.20^{b}	6.18^{b}	6.20^{b}	0.02
N-NH_3 (mg dL^{-1})	13.30	12.82	12.75	13.14	0.28
SCFA (mmol L^{-1})	1.91^{ab}	1.86^{b}	1.92^{a}	1.86^{b}	0.01
TGP 0.5 h	7.00^{a}	6.25^{ab}	5.50^{b}	5.50^{b}	0.19
TGP 1 h	8.50^{a}	8.50^{a}	7.50^{ab}	7.25^{b}	0.19
TGP 2 h	12.25^{a}	12.25^{a}	11.25^{ab}	10.25^{b}	0.26
TGP 4 h	22.00	22.25	21.50	20.25	0.30
TGP 6 h	31.75^{a}	27.75^{b}	30.25^{ab}	29.00^{b}	0.48
TGP 12 h	54.50	51.50	53.75	52.25	0.46
TGP 24 h	86.00^{ab}	83.75^{b}	86.75^{a}	84.00^{ab}	0.46
CH_4/6 h	4.36	3.93	4.29	3.87	0.10
CH_4/12 h	6.81	6.60	7.10	6.79	0.12
CH_4/24 h	12.41	10.87	12.62	12.07	0.27
B.107 mL^{-1}	19.47^{a}	17.61^{ab}	18.92^{ab}	15.64^{b}	0.54
Meth. B. 107 mL^{-1}	0.48	0.45	0.49	0.52	0.01
Ento. 103 mL^{-1}	31850.00^{b}	37187.50^{a}	32550.00^{b}	36750.00^{a}	754.21
Holo.103 mL^{-1}	3482.50^{b}	4418.75^{a}	3753.75^{ab}	3333.75^{b}	143.01

^{a,b}Different superscripts indicate statistical significance at the same row ($p<0.01$), TGP: Total gas production after (0.5, 1, 2, 4, 6, 12 and 24 h), methane concentration after (6, 12 and 24 h), pH, SCFA: Short Chain Fatty Acid, B: Rumen bacteria, Holo: Holotricha protozoa, Ento: Entodinimorpha protozoa and methanogenes bacteria

Table 3: Effect of different levels from linseed oil beads 2 (20% oil) on different rumen fermentation and microbial parameters using GPS

	Addition of linseed beads 2 levels (%DM)				
Item	Control	2	4	6	SEM
pH	6.35^{a}	6.33^{a}	6.33^{a}	6.25^{b}	0.01
N-NH_3 (mg dL^{-1})	16.01^{ab}	17.18^{a}	13.44^{ab}	12.18^{b}	0.71
SCFA (mmol L^{-1})	1.71	1.86	1.82	1.77	0.02
TGP 0.5 h	5.00	3.75	4.00	4.00	0.19
TGP 1 h	5.75	5.25	5.25	5.00	0.15
TGP 2 h	9.00	6.75	8.00	7.50	0.33
TGP 4 h	18.00	16.00	16.75	16.25	0.30
TGP 6 h	24.00	23.00	23.00	23.00	0.27
TGP 12 h	45.50	44.25	43.75	43.25	0.46
TGP 24 h	77.25	83.75	82.25	79.75	1.00
CH_4/6 h	2.48	2.69	2.63	2.65	0.10
CH_4/12 h	5.89	5.84	5.98	5.81	0.10
CH_4/24 h	12.41	11.69	12.93	12.38	0.24
B.10^7 mL^{-1}	22.86^{a}	21.00^{ab}	23.84^{a}	18.70^{b}	0.60
Ento. 10^3 mL^{-1}	19162.50^{ab}	17062.50^{b}	17150.00^{ab}	19337.50^{a}	375.97
Holo.10^3 mL^{-1}	3832.50	3832.50	3718.75	3710.00	35.90
Meth. B. 10^7 mL^{-1}	0.55	0.48	0.57	0.54	0.01

^{a,b}Different superscripts indicate statistical significance at the same row ($p<0.01$), TGP: Total gas production after (0.5, 1, 2, 4, 6, 12 and 24 h), methane concentration after (6, 12 and 24 h), pH, SCFA: Short Chain Fatty Acid, ammonia concentration (NH_4), B: Rumen bacteria, Holo: Holotricha protozoa, Ento: Entodonerma protozoa and methanogens bacteria

Table 4: Effect of treatments on rumen fermentation and microbial parameters using rustic system

	Treatments				
Item	Control	Linseed oil	Beads 1	Beads 2	±SE
pH	6.91	6.91	6.92	6.92	0.01
Red.-oks.	-236.71	-237.09	-233.91	-250.56	1.70
Flow (mL)	481.25	478.75	473.75	488.75	5.54
N-NH_3 (mg dL^{-1})	9.43^{a}	8.49^{b}	9.56^{a}	9.31^{a}	0.14
B.10^7 mL^1	24.71	22.97	24.77	25.27	0.49
Meth. B. 10^7 mL^{-1}	0.48	0.36	0.39	0.35	0.13
Ento. 10^3 mL^{-1}	1443.75^{c}	1706.25^{b}	2931.25^{a}	2581.25^{ab}	221.94
Holo.10^3 mL^{-1}	765.62^{a}	494.37^{b}	754.69^{a}	745.94^{a}	27.36
TGP (mmol g^{-1})	3402.00	3679.75	3849.12	3675.25	152.39
Methane (mmol g^{-1})	72.554	60.36	60.12	63.49	3.41
IVDMD (%)	63.74	60.17	63.06	60.68	0.65

Treatments were control, beads 1 (15% oil content), beads 2 (20% oil content) and linseed oil on 4% (DM basis), ^{a,b,c}Different superscripts indicate statistical significance at the same row ($p<0.01$)

Table 5: Effect of treatments on TVF's and VFA fraction

	Treatments				
Item	Control	Linseed oil	Beads 1	Beads 2	±SE
TVFA's	76.59	71.75	76.11	71.64	1.66
Acetate	51.14	46.90	50.22	46.90	1.23
Propionate	9.50	9.62	9.60	9.02	0.24
A/P	5.40	4.88	5.27	5.20	0.09
Isobutyrate	0.64	0.62	0.64	0.63	0.01
Butyrate	9.69	8.82	9.74	9.40	0.19
Isovalerate	2.12	2.43	2.19	2.14	0.08
Valerate	3.50	3.37	3.72	3.54	0.08

Treatments were control, linseed oil, beads 1 (15% oil content) and beads 2 (20% oil content) on 4% (DM basis), Volatile fatty acids are expressed as percentage of total

amino acid synthesis and microbial growth which it was shown with this experiment. Previous experiments with rumen fluid from sheep showed inconsistent results, with significant increases or decreases in ammonia with linolenic or linoleic sources (Gomez-Cortes *et al*., 2008; Zhang *et al*., 2008). According to Shingfield *et al*. (2008), sunflower oil supplementation tends to reduce ammonia concentration in the rumen of cattle, whereas fish oil supplementation has been reported to increase it (Keady and Mayne, 1999). The diets with sunflower and rapeseed oil showed numerically higher ammonia concentration in rumen fluids (about 1.2-3.0 mg 100 mL^{-1}) and concerning the diet with linseed oil ammonia concentration was significantly ($p<0.001$) higher incomparison to control (Jalc and Ceresnakova, 2002). In contrast, Loor *et al*. (2002) reported that ruminal ammonia-nitrogen concentration was decreased in response to fat supplementation and was lower with canolamid (canola oil treated with ethanolamine) compared with oil. Other reports indicated lower ammonia nitrogen when a control diet supplemented with linseed oil (Ikwuegbu and Sutton, 1982), rapeseed oil (Ferlay *et al*., 1993), or casein-formaldehyde-treated rapeseed oil (Tesfa *et al*., 1992) and they speculated that unsaturated oils decrease rumen ammonia-nitrogen mainly due to a decrease in rumen protozoa.

The results of Short Chain Fatty Acids (SCFA) indicated that different levels of unprotected linseed oil (Table 1) did not affect significantly ($p>0.01$) on SCFA concentrations. Beads 1 supplementation was have a significant ($p<0.01$) effect on SCFA concentration at 2 and 6% levels compared to control, however, no significant difference between 4% level and control was shown (Table 2). No significant effect ($p>0.01$) for different levels of beads 2 supplementation on SCFA was found (Table 3).

The oils rich in unsaturated fatty acids are usually considered inhibitors of rumen fermentation processes, affecting, for example, carbohydrate digestion (Doreau and Chilliard, 1997). The described effect is usually connected with depressed VFA production and lower acetate/propionate ratio. In the present trial, supplementing of plant-derived fats, protected or unprotected, did not disturb rumen fermentation in this respect. The current results are in agreement with those reported by Szumacher-Strabel *et al.* (2009). They concluded that the addition of oils rich in linoleic acid did not strongly influence rumen fermentation and up to 5% in dietary dry matter may be a valuable supplement for ruminants. Also, the changes in the rumen fermentation patterns were in agreement with physiological standards. Although, Lee *et al.* (2005) observed an inhibition of microbial activity by the highest level of fish oil causing a reduction in total VFA concentration in the rumen, other studies involving sheep (Fievez *et al.*, 2003) and cattle (Keady and Mayne, 1999; Shingfield *et al.*, 2008; Toral *et al.*, 2009) reported no significant effect of oil supplementation on total VFA concentration at any specific time post-feeding. Moreover, Sterk *et al.* (2010) studied the effects of chemically or technologically treated linseed products as different forms for fatty acids protection comparing to linseed oil in the *in vitro* scale. They reported that TVA concentration, acetate, propionate, butyrate proportions and the ratio of non-glucogenic and glucogenic VFA were not affected by treatments indicating no differences in fermentation pattern.

For gas kinetics investigation, gas production was measured at different times from incubations. The results indicated that the unprotected linseed oil did not have a significant effect ($p>0.01$) on gas production either different levels or different times. The cumulative gas production after 24 h of incubation was 76.50, 78.25, 79.25 and 79.50 mmol L^{-1} for control, 2, 4 and 6% levels of unprotected linseed oil supplementation, respectively. For beads 1 supplementation, at 0.5 h of incubation, gas production was decreased significantly by 4, 6% levels of supplementation compared to control, however, no significant difference between 2% level of beads 1 supplementation and control. At both 1 and 2 h of incubation, 6% level of beads 1 supplementation decreased gas production significantly than control, however, no significant difference ($p>0.01$) was detected among 2, 4% and control. Additionally, at 4 and 12 h of incubation, beads 1 in different levels of supplementation did not affect significantly ($p>0.01$) gas production, while, at 6 h of incubation, gas production was decreased significantly by 2 and 6% levels of beads 1 supplementation. Total gas production after an incubation of 24 h did not affect significantly ($p>0.01$) by the different levels of beads 1 supplementation and values were 86, 83.75, 86.75 and 84 mmol L^{-1} for control, 2, 4 and 6% levels of beads 1 supplementation, respectively.

The results of Table 3 indicated that there is no significance difference ($p>0.01$) among the different levels at different times and also cumulative gas production after an incubation of 24 h within beads 2 supplementation. Total gas production after an incubation of 24 h, values were 77.25, 83.75, 82.25 and 79.75 mmol L^{-1} for control, 2, 4 and 6% levels of beads 2 supplementation. Generally, there is no clear effect for linseed oil, linseed oil beads 1 and linseed oil beads 2 on rumen fermentation pattern. It may be due to the small quantities from additional levels from linseed oil and linseed oil beads and/or the short term of incubation. Ribeiro *et al.* (2005) concluded that

changes in fermentation pattern likely reflect shifts in the bacterial population in response to changes in fermentable substrates. The results of the current trial therefore indicate no shift on bacterial population for the different linseed oil forms (protected and unprotected) except the depression of bacteria count with the high level from beads 1 and beads 2. These results are in a good agreement with those reported by Sterk *et al.* (2010) who examined the effects of chemically or technologically treated linseed products and docosahexaenoic acid addition to linseed oil on biohydrogenation of C18:3n-3 *in vitro*. They stated that the overall cumulative gas production did not differ among the treatments, whereas some of the gas production curve parameters did differ between treatments, however, no clear effect was found of one of the treatments on these parameters. Moreover, Getachew *et al.* (2001) and Sinclair *et al.* (2005) did not observe differences in gas production profiles when different treatments rich in C18:3n-3 was incubated *in vitro* for 48 h. In contrast, Mohammadian-Tabrizi *et al.* (2011) found a reduction of GP over incubation time after coating wheat grain with hydrogenated tallow and hydrogenated palm oil and this may be associated with microbial attachments, whereas these unsaturated fatty acids actas toxins for rumen bacteria (Henderson, 1973; Hunter *et al.*, 1976; Kim *et al.*, 2000). It has been suggested that dietary fats may coat some nutrients and interfere with microbial attachment and depressed digestibility (Devendra and Lewis, 1974). The same results were obtained by Toral *et al.* (2009) who reported that *in vitro* gas production was slightly reduced when inocula from the animals fed the oil-rich diet were used to incubate either the control (-3.6%) or the sunflower/fish oil diet (-6.9), probably, due to changes in the rumen microbial communities produced by the oil supplementation, as previously thought.

The results of Table 1 clearly indicated lower methane production than control of the different levels of unprotected linseed oil with only significant ($p<0.01$) difference for 6% level at 6 h of incubation. After 12 and 24 h of incubation, different levels of unprotected linseed oil did not have a significant effect on methane concentration. The results of Table 2 and 3 showed that there are no significant differences ($p>0.01$) among the different levels of both beads 1 and beads 2 supplementation at the different times of incubation. Methanogens bacteria are a separate group of organisms, which are an ordinary component of the rumen microbial ecosystem. Hydrogen (H_2) and dioxide carbon (CO_2) are the principal substrates used by rumen methanogens to produce CH_4. Consequently, compounds that directly inhibit activity of methanogens are likely to reduce CH_4 and total gas production (Baker, 1999). According to this theory, the represented data (Table 2 and 3) for beads 1 and beads 2 treatments within gas production system did not show significant changes for methanogens bacteria with the different levels and thereby methane production. Nearly, similar results were shown with the linseed oil treatment except 2% level which has a significant depression on methanogens bacteria count but this depression did not have a significant effect on methane concentration within the same level. The inhibitory effect of fat supplementation on the rumen methanogenesis is determined by the source and quantity of fat used (MachMueller *et al.*, 1998; Zhang *et al.*, 2008), fatty acid composition and degree of saturation (Szumacher-Strabel *et al.*, 2009). The results of current study are in consistent with those obtained by Varadyova *et al.* (2010) who reported that methane production was not affected by soybean oil treatment. On the other hand, the depression of CH_4 production with addition of unsaturated fatty acids has been attributed to the fact that these fatty acids can serve as electron acceptors during the biohydrogenation in the rumen (Hegarty, 1999). Moreover, Van der Honing *et al.* (1981) observed a decrease of 5-10% in CH_4 produced from dairy cows fed 5% tallow or soybean oil. The reduction in CH_4 was attributed to a decrease in fermentable substrate rather than to a direct effect

on methanogenesis. However, other studies confirmed that the depression of methane production is usually accompanied by a reduction in the number of protozoa resulting from the increased dietary fat concentration (Kreuzer *et al*., 1999; Sallam *et al*., 2009).

For microbial population, total bacteria, methanogenes bacteria *Entodiniomorpha* and *Holotricha* protozoa were counted and it were represented in Table 1-3 for unprotected linseed oil, beads 1 and beads 2, respectively. The results of Table 1 indicated non significant increase of total bacteria count as the level of linseed oil up to 4%. The same total bacteria count was reported for both 4 and 6% levels. Within beads 1 treatment, total bacteria was lower in the different levels than control with significant reduction in the 6% level. Also, the total bacteria count within beads 2 treatment was significantly decreased ($p<0.01$) with 6% level supplementation compared to control, however, it was slightly increased numerically with 4% level than control but not significant ($p>0.01$).

For unprotected linseed oil, unsaturated FFA inhibits bacterial activity particularly during the lag-phase of microbial development (Maczulak *et al*., 1981), making bacteria most sensitive to the presence of FFA in the beginning of the incubation. However, during the first 24 h of incubation period of the current study, linseed oil supplementation did not change the fermentation pattern, despite the release of FFA from the triglyceride fraction (Dohme *et al*., 2001). Nevertheless, the lipolysis process might have been relatively slower in the beginning of the incubation as the bacteria of the rumen inoculum were not adapted to linseed oil. Consequently, by the time considerable amounts of FFA were released in the incubation medium, the activity of cellulolytic and methanogens might have been suppressed. The depression on bacteria count within beads 1 and beads 2 supplementation can be attributed to the relative lower pH values in the same treatments at the different levels (Table 4 and 5). They studied the effect of fish oil supplementation on rumen fermentation. They attributed the relatively low pH values to the ration of feed to incubation medium (1.6 g hay per 100 mL) which was relatively high in their *in vitro* study which might have influenced the microbial flora in terms of stimulation of amylolytic bacteria and suppression of cellulolytic and methanogens bacteria.

Moreover, the results of Table 1 indicated that the different levels of unprotected linseed decreased methanogenes bacteria compared to control with significant reduction of the 2% level. However, this depression did not have a significant effect ($p>0.01$) on methane production within the same level. The methanogens bacteria were not affected by different levels supplementation comparing to control within beads 1 (Table 4) and beads 2 (Table 5) treatments. As described before, methanogens bacteria are a separate group of organisms, which are an ordinary component of the rumen microbial ecosystem. Hydrogen (H_2) and dioxide carbon (CO_2) are the principal substrates used by rumen methanogens to produce methane CH_4. Consequently, compounds that directly inhibit activity of methanogens are likely to reduce CH_4 and total gas production (Baker, 1999). According to this theory, the represented data (Table 4 and 5) for beads 1 and beads 2 treatments within gas production system did not show a significant changes for methanogens bacteria with the different levels and thereby methane production.

Concerning to protozoa count, the results of Table 1 indicated that *Entodinimorpha* count (*Entodiniomorphid* protozoa have enzymes that attack cellulose, hemicelluloses, etc.) were lower in the different levels supplementation of unprotected linseed oil than control with significant difference ($p<0.01$) in the 4% level. *Holotricha* protozoa (*Holotricha*-depend on nonstructural polysaccharide, especially, starch and soluble sugars) also was negatively significant affected by unprotected linseed oil supplementation at 4 and 6% levels, however, no significant difference between 2% level and control was found.

For beads 1 treatment (Table 2), *Entodinimorpha* count were increased significantly ($p<0.01$) by 2% and 6% supplementation compared to control, however, there was no significant difference ($p>0.01$) between 4% level and control. Also, *Holotricha* protozoa count were increased significantly ($p<0.01$) by 2% level of beads 1 supplementation than control, however, non significant lower *Holotricha* count was found for 4, 6% level than control. Furthermore, the *Entodinimorpha* counting within beads 2 treatments (Table 3) were decreased significantly ($p<0.01$) by 2% level supplementation than control but no significant difference with 4, 6% and control was detected. On the other hand, non significant lower *Holotricha* count was found for 4 and 6% treatment than control.

It is well known that oils high in C18 fatty acids are toxic to protozoa (Newbold and Chamberlain, 1988; Ivan *et al*., 2003, 2004; Hristov *et al*., 2004). According to this theory, the results of the current trial indicate that rumen ciliates are more negatively affected than bacteria. The encapsulation process may help to avoid the negative effect of unprotected linseed oil on rumen protozoa that it was clear from the represented data (Table 3-5). These findings were also observed by Jalc and Ceresnakova (2002) who reported that reduced protozoa counts were observed using each kind of plant oils. Also, Varadyova *et al*. (2007) found that diet supplemented with 5% of linseed oil decreased the protozoan population in comparison with the control in the rumen fluid of sheep in this experiment. Szumacher-Strabel *et al*. (2008) evaluated oils differing in fatty-acid composition (rapeseed, sunflower and linseed oils). They observed that the higher the number of unsaturated bonds in fatty acids present in supplemented oils, the higher the decrease in the butyrate concentration and protozoa counts. However, Ivan *et al*. (2001) observed non significant effect of soybean oil supplementation on the protozoan concentration.

The overall results from the gas production system trial concluded that beads 1, beads 2 and linseed oil in the free form at different levels did not have a negative effect on fermentation pattern in most cases. It may be attributed to the short term of incubation and or to the low dose which was used. The only clear negative effect for linseed oil treatment was on protozoa count and it can conclude that the encapsulation process has a good benefit to avoid this negative effect for linseed oil. The closed level for control case from the three used levels was 4% which was used for the next experiments.

Rustic system

Rumen fermentation parameters: The rumen simulation technique (Rustic) enables a long-term laboratory simulation of rumen fermentation for study of the effects of additives free of the influence of the host animal (Czerkawski and Breckenridge, 1977). It has the advantage that a stable fermentation can be maintained on a time-scale sufficient to allow the study of the possible long-term adaptation that may occur over a feeding period of several weeks (Wallace *et al*., 1981). Results illustrated in Table 4 did not find any alter in fermenter fluid pH, which recorded the same value (6.9) for all treatments. The stability of pH values for all treatments within this system may be due to that artificial saliva was continuously infused using a peristaltic pump adjusted to attain a liquid dilution rate of 0.035 h (500 mL of saliva/day). This result is in an agreement with that of the same system reported by Soliva *et al*. (2004) who examined the effect of medium chain fatty acids supplementation on rumen fermentation parameters and they reported that supplementation with mixtures of C12 and C14 did not alter fermenter fluid pH, which was 6.8 on average within all treatments.

Redox potential values also were not significantly changed by treatments and the values were -236.71, -237.09, -233.91 and -250.56 for control, linseed oil beads 1 and beads 2 treatments, respectively. The redox potential measurement is used to denote the good anaerobic conditions which it required for *in vitro* experiment systems (Soliva *et al.*, 2004). No significance differences ($p>0.01$) were found for digesta flow. Ammonia concentration was significantly lower ($p>0.01$) in linseed oil treatment than control, beads 1 and beads 2 treatments whereas no significant differences ($p>0.01$) were detected between control, beads 1 and beads 2 treatments. The values of ammonia concentrations were 9.43, 8.49, 9.56 and 9.31 mg dL^{-1} for control, linseed oil beads 1 and beads 2 treatments, respectively. These results are in line with those found within short term of incubation (GPS) at the same level of linseed oil and linseed oil beads supplementation. As explained before, ammonia concentration has to be greater than the 100 mg L^{-1} (Van Soest, 1994) as optimal for the efficiency of amino acid synthesis and microbial growth and was not affected by oil supplementation. Previous experiments with rumen fluid from sheep showed inconsistent results, with significant increases (Gomez-Cortes *et al.*, 2008) or decreases (Zhang *et al.*, 2008) in ammonia with linolenic or linoleic sources. According to Shingfield *et al.* (2008), sunflower oil supplementation tends to reduce ammonia concentration in the rumen of cattle, whereas fish oil supplementation has been reported to increase it (Keady and Mayne, 1999). The significant effect on the concentrations of ammonia would indicate that N metabolism was negatively affected by linseed oil supplementation in the current study.

Concerning to microbial population within rustic system experiment, total count of bacteria did not has a significance difference between the different treatments in spite it decreased numerically by linseed oil treatment and numerically enhanced by beads 2 treatment. The values were 24.71, 22.97, 24.77 and 25.27 10^7 mL^{-1} for control, linseed oil, beads 1 and beads 2 treatments, respectively. These results were in consistent with the results of short term of incubation (GPS) at the same level of supplementation for beads 1 and beads 2 but not with linseed oil. With long term of incubation, the numerical reductions of bacteria by linseed oil supplementation may be due to that lipids are hydrolyzed extensively in the rumen by microbial lipases, releasing long-chain fatty acids that may inhibit bacterial activity. Among long-chain FA, unsaturated ones are more antimicrobial than saturated ones (Harfoot and Hazlewood, 1997). So, biohydrogenation therefore serving to protect microbes from their toxic effect which explain the biohydrogenation of long chain fatty acids within linseed oil supplementation (unpublished data).

Furthermore, methanogenes bacteria non significantly decreased in treatments than control. The values were 0.48, 0.36, 0.39 and 0.35 10^7 mL^{-1} for control, linseed oil, beads 1 and beads 2 treatments, respectively. In this context, methane concentration also decreased non significantly in the treated groups compared with control. The values were 72.55, 60.17, 60.36 and 63.49 for control, linseed oil, beads 1 and beads 2 treatments, respectively. These results have the same trend with the short term of incubation of the current study (Table 1-3). These results confirm that the reduction on methanogens and methane production were not directly related to linseed oil supplementation because the reduction was also happened with the protected oil even it was not significant ($p>0.01$) between all treatments. These results confirm the theory that lipid supplementation seems to be a promising dietary strategy (Jouany, 1994; Spears, 1996) due to toxic effects of Free Fatty Acids (FFA) on both methanogens (Prins *et al.*, 1972) and protozoa (Czerkawski *et al.*, 1975). Another theory explained the depression on methanogenesis with addition of unsaturated fatty acids has been attributed to the fact that these fatty acids can serve as electron acceptors during the biohydrogenation in the rumen (Hegarty, 1999). Moreover,

Van der Honing *et al.* (1981) observed a decrease of 5-10% in CH_4 production from dairy cows fed 5% tallow or soybean oil. The reduction in CH_4 was attributed to a decrease in fermentable substrate rather than to a direct effect on methanogenesis.

Furthermore, *Entodiniomorpha* protozoa was enhanced significantly ($p<0.01$) by linseed oil, beads 1 and beads 2 treatments compared with control treatments. Also, protected linseed oil increased *Entodiniomorpha* protozoa significantly ($p<0.01$) than unprotected linseed oil. The values were 1443.75, 1706.25, 2931.25 and 2581.25 10^3 mL^{-1} for control, linseed oil, beads 1 and beads 2 treatments, respectively. *Holotricha* protozoa was significantly ($p<0.01$) decreased by linseed oil treatment than control, beads 1 and beads 2 treatments, but no significance differences between beads 1, beads 2 and control treatments were detected. The values were, 765.62, 494.37, 754.69 and 745.94 10^3 mL^{-1} for control linseed oil, beads 1 and beads 2 treatments, respectively. These results are inconsistent with those reported within the short term of incubation in the present study (Table 1-3). The present results may give an indication that *Entodinimorpha* protozoa are become more adapted with protected linseed oil supplementation and more negatively affected by long term of incubation with unprotected linseed oil. *Holotrich* protozoa are still negatively affected by linseed oil supplementation and this negative effect was avoided with linseed oil protected form. The present results are in an agreement with those reported by Jalc and Ceresnakova (2002) who reported that protozoa counts were decreased by using different kinds of plant oils. Also, Varadyova *et al.* (2007) found that diet supplemented with 5% of LO decreased protozoan population in comparison with the control in the rumen fluid of sheep. Szumacher-Strabel *et al.* (2008) evaluated the effect of oils differing in fatty acids composition (rapeseed, sunflower and linseed oil). They observed that the higher the number of unsaturated bonds in fatty acids present in supplemented oils, the higher the decrease in the butyrate concentration and protozoa counts. In contrary, Ivan *et al.* (2001) reported no significant effect of soybean oil supplementation on the protozoan concentration.

Total gas production (Table 4) did not have a significance difference ($p>0.01$) between treatments. The values were, 3402, 3679.75, 3849.12 and 3675.25 mmol g^{-1} OM for control, linseed oil, beads 1, beads 2 and treatments, respectively. These results are in an agreement with those of Getachew *et al.* (2001) and Sinclair *et al.* (2005). They did not observe differences in gas production profiles when different treatments rich in C18:3n-3 were incubated *in vitro* for 48 h. In contrast, Mohammadian-Tabrizi *et al.* (2011) found a reduction of GP over incubation time after coating wheat grain with hydrogenated tallow and hydrogenated palm oil and this may be associated with microbial attachments, whereas these unsaturated fatty acids actas toxins for rumen bacteria (Henderson, 1973; Hunter *et al.*, 1976; Kim *et al.*, 2000).

No significance difference ($p>0.01$) was found for *In Vitro* Dry Matter Disappearance (IVDMD) between treatments. The values were 63.74, 63.06, 60.68 and 60.17% for control linseed oil beads 1 and beads 2 treatments, respectively. The digestibility of dry matter was determined by the percentage of DM disappearing from the fermentation bottles during 48 h of incubation. The present results are in consisting with those reported by Toral *et al.* (2009). They reported that ruminal degradation of DM, CP and NDF was not affected by a combination of soybean and fish oil supplementation. Also, in agreement results were obtained by Keady and Mayne (1999), who observed no effect of fish oil supplementation on IVDMD, even when a shift in the rumen fermentation pattern was observed. In the present study, although lipid supplementation could have limited the number of predominant cellulolytic bacteria, which are usually more affected by oil supplementation (Doreau and Chilliard, 1997), other microbial population, able to degrade fiber,

might have occupied their niches, which would explain the absence of any effect on the degradation of fiber. In contrast, several experiments showed strong negative effects of 50-70 g kg^{-1} diet Dry Matter (DM) linseed oil on ruminal digestion in sheep (Ikwuegbu and Sutton, 1982; Sutton *et al*., 1983). This effect was ascribed partly to a large drop in the protozoa population.

Total and fractions of volatile fatty acids: The results of volatile fatty acids, TVFA's and fraction, are illustrated in Table 5. The total volatile fatty acids, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid were measured and A/P ratio was calculated. From statistical analysis, no significant differences ($p>0.01$) between all treatments with TVFA's and VF fraction were found. The results noted that acetic acid and TVFA's were decreased by linseed oil and beads 2 treatments numerically but not significant ($p>0.01$). This decline, even it was not significant, was in a line with a decreasing on IVDMD values, protozoa and bacteria count in the same treatments.

The present results are in agreement with those of Fievez *et al*. (2003) in sheep and Keady and Mayne (1999) and Shingfield *et al*. (2008) on cattle who reported no significant effect of oil supplementation on total VFA's concentration. Moreover, Toral *et al*. (2009) reported that total VFA concentration was not affected at any specific time post-feeding and only its mean value showed a trend ($p = 0.098$) to be lower with oil supplementation. Concerning particular VFA, the propionate concentration was not significantly changed in this study, whereas the average acetate concentration showed a reduction with oil supplementation. This suggests that acetate-producing bacteria, such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, which are considered to be predominant cellulolytic bacteria in the rumen, may have been more inhibited by PUFA (Maia *et al*., 2007; Zhang *et al*., 2008). The addition of fish oil to the diet has often been reported to result in an increase in the molar proportion of propionate concentration and a decrease in acetate (Doreau and Chilliard, 1997; Keady and Mayne, 1999; Wachira *et al*., 2000; Fievez *et al*., 2003). Similar results were observed when linoleic acid was incubated *in vitro* with rumen fluid from sheep (Zhang *et al*., 2008), whereas supplementation with sunflower oil in cattle did not affect the rumen VFA proportions (Beauchemin *et al*., 2007a). From a physiological point of view, a shift in the rumen microbial communities may result in changes in biohydrogenation and consequently, in the milk FA profile (Palmquist *et al*., 2005). Data of Table 5 clearly indicated non significant effect of treatments on butyrate. The effect of unprotected linseed oil supplementation is very inconsistent, with reductions with fish oil or linoleic acid (Fievez *et al*., 2003; Zhang *et al*., 2008), no effects with linoleic-rich sources or fish oil (Keady and Mayne, 1999; Beauchemin *et al*., 2007b; Shingfield *et al*., 2008) and even increases with a combination of sunflower oil and fish oil (Palmquist and Griinari, 2006) having been reported. Reduced butyric concentrations are usually associated with reduced protozoa numbers and also with their depressed activity (Szumacher-Strabel *et al*., 2009) and the obtained results confirm this hypothesis. Valerate and branched-chain VFA, originated from the deamination of some amino acids, were not affect by linseed oil and linseed oil beads supplementation. Szumacher-Strabel *et al*. (2009) found that borage oil and Saint-Mary thistle oil decreased the level of isobutyrate in dairy cows rumen fluid after incubation, also, Saint-Mary thistle oil supplementation decreased the level of isovalerate. According to Wolin *et al*. (1997) these changes may also be connected with decreased of ruminal microbe numbers and reduced proteolysis.

Concerning to A/P ratio, results of Table 5 indicated non-significant lowering for A/P ratio with unprotected linseed oil than control. This result is due to the decline in acetate portion within the

same treatment. Acetic acid levels can drop if there is a lack of effective fiber in the ration or high intakes of oil can also depress acetic acid which (Szumacher-Strabel *et al.*, 2011) which it in consist with the present study.

In conclusion, the fatty acids, omega 3 and omega 6 in particular had some negative effects on rumen fermentation parameters and microbes. Encapsulation of linseed oil using alginate and k-carrageenan polymers was able to avoid these negative effects. *In vivo* studies on ruminant animals should be conducted in future to confirm these results.

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