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Original Research Article

Fish oil and *Leucaena leucocephala* in methane production during *in vitro* fermentation of king grass CT-115

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Keywords

Fish oil, *Leucaena leucocephala*, King grass CT-115, Methane production, Fermentation *in vitro*, Ruminants

A B S T R A C T

The objective of this study was determine the effect of adding fish oil, *Leucaena leucocephala* and concentrate feed, on a diet of pasture King-grass CT-115 during rumen fermentation *in vitro*. The treatments were: T1= 100% pasture; T2= 99.5% pasture + 0.5% fish oil; T3= 95% pasture + 5% fish oil; T4= 70% pasture + 30% of *L. leucocephala* and T5= 60% pasture + 40% concentrate. Variables evaluated were: pH, VFA concentration, total bacteria, cellulolytic bacteria and protozoa also CH_4 production and IVDMD. The results were analyzed by ANOVA in a completely randomized model. Lower pH to 6.2 in T1, T4 and T5 was obtained, the VFA concentration was higher ($P \leq 0.05$) in T2, which increased the ratio of acetate:propionate. There was a smaller population ($P \leq 0.05$) total bacteria in T3, T4 and T5; protozoan population was higher in T3 and T5. CH_4 production was higher in T2, and lower ($P \leq 0.05$) in T3 and T5; there were no differences in IVDMD between treatments. It was concluded that the addition of fish oil and *L. leucocephala* on a diet of King Grass CT-115, did not affect the populations of total and cellulolytic bacteria; incorporation of 5% fish oil and 40% of concentrate decreased methane production in the ration.

Introduction

There is a large concern about the contribution of ruminants to global warming, because they produce greenhouse gases (GHG) such as methane (CH_4) and carbon dioxide (CO_2), which are by-products of rumen fermentation (Moss *et al.*, 2000; Eckard *et al.*, 2010).

In Mexico, according to the national inventory of greenhouse gases in 2009, 85% of total CH_4 emissions from agriculture come from farming, and of this percentage, 89% is produced by cattle (Ordonez and Hernandez, 2008). In the tropics, the feeding of ruminants is based on forages containing

low nutrient, a high proportion of cell walls and low digestibility; such conditions result in increased production of CH_4, which can represent 15 to 18% of the digestible energy consumed by the ruminant (Kurihara *et al.,* 1999). It is estimated that 90% of CH_4 produced in the rumen and the rest in the large intestine by methanogenic Archaea group of organisms that use H_2 to reduce CO_2 and CH_4 and synthesize ATP as final products (Lana *et al.,* 1998; Rusell and Richlik, 2001; Deramus *et al.,* 2003). Reduce CH_4 production in ruminants is a challenge, because by decreasing the production of gas, the efficiency of energy utilization is improved diet, which can lead to economic and environmental benefits (Teferedegne, 2000). Have been developed different strategies to reduce methane emissions; the most practical and efficient, is manipulation of diet to change patterns of fermentation using forages improved nutritional quality, including a high proportion of grains in the diet, have also been used additives such as organic acids and ionophores which reduce acetate to propionate ratio, reducing CH_4 production in rumen (Bhatta *et al.,* 2009). Another method that reduces CH_4 production in ruminants, are acetogenic bacteria inocula (McAllister *et al*,. 1996; Baker, 1999), which use hydrogen ions and causing them to be metabolized as propionic acid, decreasing production of CH_4 and improves efficiency of energy use (Boadi *et al.,* 2004).

It has also been added to the diets vegetable oil or animal fat, which compete for the hydrogen ions with methanogenic microorganisms in rumen fermentation (Czerkawski *et al.,* 1966); which decreases the concentration of protozoa and has a toxic effect on methanogenic microorganisms. In recent years they have been added to the diet of ruminants in the tropics, legumes and arboreal, which are an alternative sustainable and friendly to environment (Tiemann *et al.,* 2008). *Leucaena leucocephala* has been used as desfaunante agent due to its content of secondary metabolites that are toxic to protozoa, which produce lower emissions of CH_4 (Gurbuz, 2009); also has the advantage that it can be used in diet as a protein supplement. Moreover, it is considered that an increase in proportion of concentrate up to 50% in ruminant diets, reduces the growth of protozoa (Act, 2010), thus proliferation methanogenic microorganisms is reduced, which results in a decrease in CH_4 emissions; however, this practice is costly for production systems in the tropics (Clemens and Ahlgrimm, 2001; Santacoloma, 2011).

In research conducted to reduce CH_4 emissions in ruminants, have been incorporated into the diets vegetable oils, animal fats, leguminous trees and a high proportion concentrated food. So far, the generated information is limited to measuring CH_4 and some parameters of rumen fermentation; sometimes conflicting results have been reported, and in general, have not been explained concretely the effects of the addition of the foregoing ingredients in diets based on tropical forages; particularly on pH, ruminal fermentation parameters, the concentration of rumen microbial populations, CH_4 production and *in vitro* dry matter digestibility (IVDMD).

The objective of this research was to determine the effect of the addition of fish oil, *Leucaena leucocephala* or supplement; on pH, VFA concentration, total population of bacteria, cellulolytic bacteria, total protozoa; CH_4 production, and IVDMD; on a diet made King grass CT-115 pasture during *in vitro* rumen fermentation.

Materials and Methods

Study Area

The study was conducted in the Laboratory of Biochemistry and Nutrition, Universidad del Mar, Campus Puerto Escondido, Oaxaca, Mexico. Chemical analyzes of samples were performed in the laboratory of Rumen Microbiology and Microbial Genetics belonging to the Colegio de Postgraduados, Montecillo, Texcoco, Mexico.

Experimental treatments

Five experimental treatments were evaluated; T1 = 100% King grass CT-115 pasture; T2 = 99.5% King grass CT-115 pasture + 0.5% fish oil; T3 = 95% King grass CT-115 pasture + 5.0% fish oil; T4 = 70% King grass CT-115 pasture + 30% *Leucaena leucocephala*; T5 = 60% King grass CT-115 pasture + 40% concentrate. The King grass CT-115 pasture were collected at 90 d of growth in an experimental plot established in the experimental field of the Universidad del Mar campus Puerto Escondido, Oaxaca.

Experimental procedure

The experiment was conducted under conditions *in vitro,* using the method reported by Rodriguez (2009), gas trapping. They were used 15 serological glass vials with capacity of 100 mL, as biodigesters. Under anaerobic conditions were added to each vial 45 mL of a culture medium glucose-cellobiose and starch, using as basis clarified rumen fluid (GCA-FR), according to the methodology described by Cobos and Yokoyama (1995). The vials were sealed with rubber stoppers and aluminum-rings, sterilized at 121°C and 1.5 atm for 15 min, then were tested for sterility. A cow Brown Swiss x Zebu rumen cannulated, stabled and fed for 21 d with a diet of grass and King Grass CT-115 water ad libitum were used for obtaining the ruminal fluid. The rumen fluid was removed at 08:30 h, filtered in cheesecloth four capable, it was subsequently deposited in a hermetically sealed thermo were transported to the laboratory, and was deposited in a round-bottomed flask with CO_2 flux at 39°C until use, which occurred 50 min after collection.

The inoculated digesters were placed in a water bath RIOSSA trademark, model BMME at 39°C; Each digester was coupled to a trap with acidic saline solution, prepared according to the method reported by Baez (2010). For trapping the biogas produced, Taygon® hoses, provided at their ends with hypodermic needles were used. In traps a relief valve adapted to equalize the atmospheric pressure. Finally, the gas trap was placed in a graduated cylinder upside down to 50 mL, to measure the displacement of acid trap saline solution.

Determination of pH

The pH was determined after 72 h of incubation (Lana *et al.,* 1998) in the different treatments with a potentiometer trademark HANNA® HI2210 model calibrated at two points (4.0 and 7.0).

Concentration of VFA

For determination of the AGV, the method of phenol-hypochlorite (McCullough, 1967) was used; 1 mL of each sample was transferred with 0.25 mL of 25% metaphosphoric acid in Eppendorf tubes with a capacity of 2.0 mL. In an Eppendorf 5810R centrifuge, were centrifuged at 11,000 rpm for 10 min at 4°C. Thereafter the supernatant was taken and placed in vials for chromatography. The VFA concentration was determined after 72 h incubation in a gas chromatograph Clarus Perkin Elmer® model 500 with autosampler

and Elite FFAP capillary column, hydrogen was used as carrier gas with a flow of 15 mL min-1, the injector temperature, detector, and heater was 200, 250 and 140 °C, respectively.

Microbiological Analysis

Total bacteria concentration was determined at 0, 6, 12, 24, 48 and 72 h of incubation (Stewart *et al.,* 1997), by the direct method in a Neubauer-improved camera and an optical microscope MOTIC® trademark, model DMB3, at a magnification of 100X. The concentration of bacteria per mL^{-1} of rumen fluid was calculated as the product of the average of counted cells for the dilution factor in the constant of the chamber (50,000).

Cellulolytic bacteria concentration was estimated by the most probable number method (MPN, Harrigan and McCance, 1979), after 72 h of incubation in culture tubes with triplicate samples, provided with an anaerobic liquid medium prepared according to the methodology cited by Cobos *et al.* (2002).

Cellulolytic bacteria development was confirmed by degradation of Whatman 541 paper after 10 d of incubation at 39°C. Ruminal protozoa concentration was determined by direct count using a Neubaer-improved camera, and with an optical microscope MOTIC® trademark, model DMB3, at a magnification of 40X, protozoa concentration was expressed by mL^{-1} of ruminal fluid, as the average product protozoa in a volume of 1 x 1 x 0.1 mm multiplied by the dilution factor.

Production of CO_2 and CH_4

A record of the volume of gas produced by the acid salt solution was made, by volume displacement of the traps at 0, 6, 12, 24, 48 and 72 h of incubation (Rodriguez, 2009). The gas captured in traps was subjected to thermal conductivity by gas chromatography to determine the percentages of CH_4 and CO_2. A gas chromatograph Clarus 500 Perkin Elmer® model was used, with thermal conductivity detector and a column PE 6'x1 / 8 ODSS: Propak 080/100 and manually injected 0.1 mL of sample. The characteristics and conditions of the method were: a) temperature ramp in the oven start 28°C min^{-1} ramp 2.5°C min^{-1}, the final 80°C 0.5 min^{-1}; b) TCD injector temperature 130°C; c) 0.1 mL injection volume, d) flow of carrier gas (helium) 23.5 mL min^{-1}, e) CH_4 retention time was 1 min ± 0.05, and CO_2, 2 min ± 0.05.

In vitro dry matter digestibility

At 72 h of incubation, the contents of the digester were filtered using filtration equipment vacuum pump GAST®, DOA-P704-AA model; the residue was dried in a stove FELISA® 75°C for 48 h. An analytical balance was used for obtaining constant weight. Digestibility of dry matter (DM) was determined by the method proposed by Mellenberger *et al.* (1970).

Statistical Analysis

For the analysis of the results was used a completely randomized design, were considered as experimental units biodigesters and were randomly assigned to one of five treatments with three replications. The data were analyzed by analysis of variance using the PROC GLM procedure of SAS (2010) and the Tukey test was used to compare means (Steel and Torrie, 1988). Variables for total bacterial concentration, cellulolytic bacteria and protozoa, a logarithmic transformation were performed to normalize the data.

Results and Discussion

Values of pH and VFA

The pH value at T2 was higher (P <0.05) than the other treatments (Table 1); while T3 had a pH of 6.55 which was higher (P <0.05) at T1, T2, T4 and T5, which had values of 6.24, 6.12, 6.16 and 6.09, respectively. Total VFA production was higher (P <0.05) in T2 compared with T1, T3 and T4; being similar to T5 (Table 1). The acetate concentration was higher (P <0.05) in T2 and T4; while T5 present the lowest concentration (P <0.05). Furthermore, the propionic acid concentration was higher (P <0.05) in T5; in contrast, the lowest concentration (P <0.05) was obtained in T2. The butyrate concentration was higher in T2, and the lowest concentration was in T5. The acetate to propionate ratio was higher (P <0.05) in T2 and the lowest ratio (P <0.05) was obtained in T5.

The average pH value between treatments was 6.23 at 72 hours of incubation, indicating that proper conditions are maintained for the activity of cellulolytic bacteria and methanogenic microorganisms (Yokoyama and Johnson, 1988). However, T5 had a pH value of 6.09 and it is likely that development of cellulolytic bacteria have been affected; as indicated by Russell and Wilson (1996) who observed a decrease in the degradation of cellulose when the pH dropped below 6.2. Shriver *et al.* (1986) and Hoover (1986) observed that a moderate decrease in pH weakens the adhesion of cellulolytic forage particles, thus reducing their degradation bacteria. Russell and Dombrowski (1980), attributed to the low concentration of cellulolytic bacteria in rumen pH unfavorable, is due to increased energy costs of maintenance and damage to the cell membrane.

Incorporating fish oil in the diet, observed during fermentation occurred in T2 greater amount of total VFA, with higher production of acetate and butyrate, which increased the ethyl propionate ratio. Martinez (2011), mentioned that the inclusion of non-protected fat sources in the diet modifies the concentrations and molar proportions of VFA. In other studies, no effect was found in the ruminal concentrations of acetate, propionate and butyrate to include some source of fat in the diet (Beauchemin *et al.,* 2007); found not effect of fat on the acetate to propionate ratio (Avila et al 2000; Ueda et al 2003; Montgomery et al 2008). In contrast, Mohammed *et al.* (2004) by adding sea horse oil to the diet reported increased concentration of propionate and decreased concentration of acetate and butyrate. In the present study, increases in the concentration of acetate and butyrate was probably due to partial increased the concentration of cellulolytic bacteria; with intense degradation of cellulose and fermentation of soluble carbohydrates by saccharolytic bacteria, producing a high concentration of acetate (Owens and Goetsch 1988). In T3 only differences in the concentration of butyrate were found, these results are consistent with Dong *et al.* (1997) who added 10% fish oil diet during fermentation and found differences in the concentration of butyrate; T3 in the acetate to propionate ratio showed a slight decrease from T1; in this context Doreau and Chilliard (1997) mentioned that the addition of dietary lipids, produces reductions in fiber digestion, with increase in the concentration of propionic acid and acetic acid decreased and butyric acids. No effects have been obtained by adding less than 5% of dietary fat. In the present study, with the addition of *Leucaena leucocephala*, no statistical differences were found in total VFA concentration or the

amount of propionate or in acetate to propionate ratio; however, were obtained decrease in the proportion of butyrate and increased production of acetate compared to the control group (T1). In contrast, Galindo *et al.* (2007) found no differences in the concentrations of acetate, propionate and butyrate, adding 30% *Leucaena leucocephala* on a diet of grass. Moreover, Estrada-Liévano *et al.* (2009) reported an increase in the production of propionate and butyrate, with the addition of *Leucaena leucocepala* a star grass diet. Rodriguez (2010) formulated a diet under conditions similar to ones of this study, added *Leucaena leucoccephala* and found no significant differences in total VFA production, or in the proportions of acetate, propionate and butyrate. Rodriguez (2010) reported that VFA production of tree legumes depends on the chemical nature of the tannins, including its structure, degree of polymerization and reactivity; in addition to its concentration in the feed. As forage legume with moderate or high amounts of tannins, tend to have lower proportions of acetic: propionic acid than those with low levels of tannins (Mbugua *et al.,* 2008; Garcia *et al.,* 2008.). By adding 40% of concentrate feed in the diet (T5), no differences in total VFA concentration was found; however, the acetate concentration was lower; whereas propionate proportion was higher than the other treatments. In this respect Bedolla (2010) mention that when the addition of concentrate not exceeding 35% of the dry matter of the diet, there is an increase in total VFA concentrations, and cellulose digestion is improved without altering the pH of rumen. When the amount of concentrate in diets containing forage is increased, an increase also occurs in the production of total VFA and propionate, with a decrease in the concentration of acetate (Beauchemin and McGinn 2005; McGeough *et al.,* 2010). Fahey and Berger (1988) mentioned that greater concentration of propionate in the rumen occurs when the ratio falls forage: concentrate; which can result in reducing methane production, because the formation of hydrogen retains propionate, which reduces the production of CH_4 (Mendoza-Martinez *et al.* 2008).

Concentration of total bacteria, cellulolytic bacteria and protozoa

The concentration of total bacteria at 72 h, at T1 and T2, were higher ($P < 0.05$) at T3, T4 and T5 (Table 2). The concentration of cellulolytic bacteria ranged from 4.96×10^6 mL^{-1} to 29.66×10^6 mL^{-1} without significant differences ($P > 0.05$). The concentration of protozoa at 72 h was higher ($P < 0.05$) in T3 and T5 compared to T1, but similar to T2 and T4 (Table 2).

The concentration of total bacteria in all treatments was maintained at 10^9 cells per mL of culture medium being within the normal range (Yokoyama and Johnson, 1988). T2, where 0.5% added fish oil in the diet, an increase in the number of total bacteria was introduced, being similar to T1; these results are consistent with data reported by Dong *et al.* (1997) who added 10% cod liver oil in a hay-based diet, and found a higher concentration of total bacteria. Moreover, Galindo *et al.* (2009), by adding 7.5% coconut oil, found no differences in the concentration of total bacteria; in this sense Machmüller and Kreuzer (1998), by adding 7% coconut oil observed an increase in the number of total bacteria. This occurs when polyunsaturated fatty acids are rapidly hydrogenated in the rumen, and coconut oil supplied in small amounts, can potentiate the growth of cellulolytic bacteria (Martinez, 2011). A different effect was observed in T3 to add 5% fish oil, wherein the total bacterial number declined ($P < 0.05$) at 72 h versus

T1. In a study by Dohme *et al.* (2003) observed that high levels of EPA and DHA in fish oils, decrease lipolysis and biohydrogenation, causing a toxic effect when the oleic acid on the surface of bacteria adhering, preventing the entry of nutrients into the cell. However, in this study there was no difference in the concentration of cellulolytic bacteria in the different treatments. Other studies have reported a decrease in the population of cellulolytic bacteria by adding oils to the diet (Maczulak *et al.,* 1981), the main causes were increasing degree of unsaturation and the presence of free carboxyl groups; however, Chalupa *et al.* (1984) mentioned that concentrations of 3-5% oil can be tolerated by rumen microorganisms. Furthermore, Dong *et al.* (1997) reported that the effect of oil on the cellulolytic bacteria population depends more on the individual properties of the fatty acids of the degree of unsaturation. In the present study the concentration of cellulolytic bacteria was not affected by the addition of oil to the diet, which can be explained by the investigations of Smith and Harris (1993), who reported that a high forage diet decreases the toxic effect of the fat on ruminal bacteria.

In diets where fish oil (T2 and T3) were added, the viability remained protozoan 72 h; These results contrast with the data reported by Machmüller and Kreuzer (1998), who added oils to the diet, and obtained a reduction in the number of protozoa; meanwhile, Yang *et al.* (2009), included in a diet for cows 4% soybean oil, 4% of linseed oil and 4% of a mixture of both, and observed a decrease in the concentration of protozoans. Zapata *et al.* (2012) reported that involvement of protozoa in the biohydrogenation is initially oriented lipolysis and subsequently the isomerization of some compounds. In this study, the increased viability of the protozoa in the diet with 5% of fish oil (T3) is added, can be explained by the protozoa are able to degrade from 50 to 85% of EPA and DHA fatty acids highly toxic to bacteria, allowing the development of the latter.

In this investigation was a decrease in the concentration of total bacteria by adding to the diet 30% of the legume *Leucaena leucocephala* (T4). Similar results have been reported by Galindo *et al.* (2003), who added the same amount of the legume tree on a diet of forage and reported a decrease in the number of total bacteria in comparison with 100% diet fodder. Moreover, Galindo *et al.* (2008) reported a linear decrease with increasing the proportion of legume in the diet; they found fewer total bacteria to incorporate up to 30% of this legume. In this study, the addition of *Leucaena leucocephala* in the diet had no effect on the concentration of cellulolytic bacteria, so there were no differences between T4 and T1; which agrees with the results reported by Galindo *et al.* (2007) and Galindo *et al.* (2008), who found no difference in the number of cellulolytic bacteria by adding 30% of *Leucaena leucocephala* on a diet based on star grass. In T3, where 30% of *Leucaena leucocephala* was incorporated, the viability of protozoa remained 72 h; results agree with previous data obtained by Galindo *et al.* (2003) who observed an increase in the concentration of protozoa, which was attributed to factors related to the legume, such as vegetative state, age, phenology, the time of year; also has been demonstrated that when some tropical species tannins have little ability to precipitate proteins may be safe for some rumen microbes (Makkar and Becker 1998). At T5 where the commercial feed in 40% of the diet was included, it decreased the concentration of total bacteria compared to T1 and T2; this can be explained by the decrease in pH to 9.6 at 72

h incubation. Wool *et al.* (1998) mentioned about that in a ruminal pH below 6.2 can affect the microbial population. Moreover, the concentration of cellulolytic bacteria in T5 did not differ (P> 0.05) compared to other treatments; In contrast, other studies have reported that diets high in concentrate, where acid production is high, a reduction is observed in the population of cellulolytic bacteria (Van Soest, 1982). However, many amylolytic bacteria (strains of *Fibrobacter succinogenes, Prevotella ruminicola, Butirivibrio fibrisolvens*) are also cellulolytic (Hungate, 1966), so the cellulolytic populations can resist moderate fluctuations in pH with slight decrease in its activity (Calsamiglia *et al.,* 2002, Hoover, 1986); this may explain why no differences between T5 and other treatments. Moreover, T5 showed an increase in the concentration of protozoans. Yokoyama and Johnson (1988) mention that diets with higher feed concentrate, increase the concentration of total protozoa; then it is important to consider that when concentrated food is included in the diet of ruminants in more than 70-80% of the MS, the pH drops below 6.0as a consequence drastically reduces the concentration of protozoa (Dennis *et al.,* 1983). It has been reported that the increase in the population of protozoa has some benefits for ruminant to avoid drastic changes in pH, because the protozoa are predatory effect on the population of rumen bacteria, total, cellulolytic and amylolytic (Mendoza *et al.,* 1993), this may explain the decrease of the concentration of total bacteria in the present study.

Production of CO_2 and CH_4 in the treatments and determination of IVDMD

CO_2 production was higher (P <0.05) in T3 (Table 3) relative to the other treatments, recording the lowest numerical value in T2; Moreover, the CH_4 concentration was higher (P <0.05) in T2 (Table 3); in contrast, the lowest concentration (P <0.05) was found in T5 and T3. By adjusting the concentration of CH_4 with the values of IVDMD was determined that the highest concentration (P <0.05) of CH_4 was presented at T2, and the lowest concentration (P <0.05) was recorded in T5.

Expressed in percent IVDMD showed no significant difference (P> 0.05) between treatments, the average value of IVDMD was 49.55% (Table 3).

The average IVDMD of King Grass CT-115 obtained during this experiment was 9.5%, which is considered relatively high for a tropical grass, considering the content of cell walls (Herrera 1997) this could be one of the factors why that the addition of fish oil (T2 and T3), *Leucaena leucocephala* (T4) and concentrated food (T5) did not differ from control group (T1).

Increased production of CH_4 was observed by adding 0.5% fish oil (T2), which contrasts with other research. Beauchemin *et al.* (2007) reported 17% reduction in methane production by adding sunflower oil, tallow and sunflower seeds, attributing a decrease in fiber digestibility. Machmüller and Kreuzer (1998) also observed decreased CH_4 production when added to the diet 3.5% and 7% coconut oil, these researchers concluded that the reduced production of CH4 is due to lower fiber digestibility and development as a minor number of methanogens and protozoa microorganisms. Increased production of CO_2 and CH_4 concentration less occurred when adding 5.0% fish oil, this is due to the decrease CH4 production, the amount of free CO_2 in the medium increases (Baez, 2010). The results of the percentage of IVDMD in the present study, in contrast to other research reports because no statistical differences (P> 0.05) on *in vitro* digestibility of dry matter, or number of protozoa between treatments

were obtained. Meanwhile, Mohammed *et al.* (2004) obtained similar to those of the present research results; adding sea horse oil in the diet, they reported an increase in the number of total bacteria, cellulolytic bacteria and protozoa, and did not differ from the control treatment. The results of this study suggest that the increased production of CH_4 in T2 can be attributed to a slight increase in the concentration of cellulolytic bacteria, which may explain the increase in the concentration of acetate, which is used as a substrate for CH_4 production (Mendoza-Martinez *et al.* 2008). The results obtained by T3 of this study are consistent with data reported by Swainson *et al.* (2007), who found no significant decrease in the production of CH_4 by adding 3% coconut oil on a diet of fresh forage *Loliumperenne* or *Cichoriumintybus*; however, numerous studies report a significant decrease in the production of CH_4 by adding oils to the diet. Dong *et al.* (1997) emphasize that the biohydrogenation alone cannot explain the reduction in methane production; coat with oil ration and subsequent lower bacterial adhesion is not responsible for the decreased production of CH_4 in the diet. Therefore, this decrease is due to a change in the fermentation towards greater production of propionate and oil has a toxic effect on the methanogenic population of microorganisms. This may account for the slight reduction in CH_4 production obtained in T3, with the further decrease in the concentration of acetate and butyrate. The results obtained in T4 are different from reports of Sallam *et al.* (2010), who report a decrease in the production of CH_4; Instead, Rodriguez (2010), by adding tannins from *L. leucocephala* during fermentation of forage King grass CT-115, no statistical differences observed. The differences between the present research, with regard to other studies, may be by different ecotypes of *L. leucocephala*, which vary in the content of anti-nutritional factors (Fortes *et al.*, 2003). In this context Ley (2010), mentioned that sometimes the secondary metabolites of certain leguminous trees have no toxic effect on rumen microorganisms, and some of them may have an increase in their concentration. T1 in the present study did not register increase in CH_4 production; this may be related to the quality of the delivered food. It has been shown that when ruminants are fed diets with high proportions of forage high in cell walls and low digestibility, the greater amount of acetic acid and H_2 is generated (Janssen 2010); which increases the amount of CH_4 produced per unit of food eaten. By contrast, in diets containing a higher proportion of easily fermentable carbohydrates, the main product is propionic acid (Beauchemin and McGinn, 2005). In a study by Boadiand Wittenberg (2002), by providing forage with high (61.5%), medium (50.7%) and low (38.5%) digestibility found that CH_4 production increased as forage digestibility was reduced; while Swainson *et al.* (2007) mentioned that it is possible to significantly reduce the production of CH_4 in forage-based diets, with higher digestibility of 50%. McGeugh *et al.* (2010) reported that increasing the ratio of concentrate and grains in the diet caused a decrease in CH_4 emissions. Lopez *et al.* (2011) observed that this reduction is affected by the source of dietary starch; Beauchemin and McGinn (2005) found that the substitution of barley grain for corn grain reduced CH_4 emissions. Miramontes *et al.* (2010) mentioned that the proportion of 40:60 forages: concentrate in the diet, allows the growth of acetogenic bacteria in the rumen, with an increase in propionate production, which explains the decrease in CH_4 production. Moss *et al.* (2000) indicated that the formation of propionate is considered competitively use of H_2 in the rumen.

Table.1 Valuesof pH and VFA concentration during ruminal fermentation *in vitro* after 72 h of incubation in diets formulated with pasture King grass CT-115, fish oil, *Leucaena leucocephala* and concentrate

Properties	Treatments					
	T1	T2	T3	T4	T5	SEM+
pH	6.12[c]	6.55[a]	6.24[b]	6.16[c]	6.09[c]	0.016
Total VFA++ mol L^{-1}	115.40[b]	125.70[a]	114.00[b]	110.97[b]	119.94[ab]	2.145
Acetate%	57.32[b]	61.21[a]	57.12[b]	59.16[ab]	54.15[c]	0.518
Propionate%	33.48[b]	25.62[c]	34.03[b]	31.99[b]	37.83[a]	0.538
Butyrate%	9.20[b]	13.17[a]	8.85[c]	8.85[c]	8.02[d]	0.059
Relationshipacetate: propionate	1.71[b]	2.39[a]	1.68[b]	1.85[b]	1.43[c]	0.042

abc Values with different superscript in a row are statistically (P <0.05) different.
T1 = 100% King grass forage Ct-115 (Control).
T2 = 99.5% King grass forage Ct-115 plus 0.5% fish oil.
T3 = 95% king grass forage Ct-115 plus 5% fish oil.
T4 = 70% king grass forage Ct-115plus 30*% Leucaena leucocephala*.
T5 = 60% king grass forage Ct-115 plus 40% concentrate.
+ SEM = standard error of the mean.
++AGV = total volatile fatty acids.

Table.2 Concentration of total bacteria, cellulolytic bacteria and protozoa in diets formulated with pasture King grass CT-115 added with fish oil, *Leucaena leucocephala* and concentrate feed during rumen fermentation *in vitro* at 72 h of incubation

Microorganisms (mL^{-1} of rumen fluid)	Treatments					
	T1	T2	T3	T4	T5	SEM+
Total bacteria (10^9 mL^{-1})	24.17[a]	31.75[a]	15.75[b]	14.75[b]	11.50[b]	1.8x10^9
Cellulolytic bacteria (10^6 mL^{-1})	7.93[a]	29.66[a]	4.96[a]	5.66[a]	3.78[a]	0.35
Protozoa (10^6 mL^{-1})	0.00[b]	1.66[ab]	5.00[a]	1.66[ab]	10.00[a]	1.6x10^6

[abc]Values with different superscript in a row are significantly different (P <0.05).
T1 = 100% forage King grass Ct-115 (Control).
T2 = 99.5% forage King grass Ct-115 plus 0.5% fish oil.
T3 = 95% forage King grass Ct-115 plus 5% fish oil.
T4 = 70% forage King grass Ct-115's 30% *Leucaena leucocephala*.
T5 = 60% forage King grass Ct-115 plus 40% concentrate.
+ SEM = standard error of the mean.

Table.3 Production of CO_2 and CH_4 (mmol) in diets with pasture King grass CT-115 added with fish oil, *Leucaena leucocephala* and concentrate during rumen fermentation *in vitro* and determination of IVDMD

Properties	Treatments					
	T1	T2	T3	T4	T5	SEM[+]
CO_2concentration (mmol mL^{-1})	58.65[b]	57.40[b]	71.35[a]	56.43[b]	58.24[b]	1.080
CH^4 concentration (mmol mL^{-1})	28.69[b]	38.70[a]	27.92[bc]	28.86[b]	25.94[c]	0.589
CH_4concentration (mmol g^{-1})	58.17[b]	78.61[a]	59.31[b]	58.88[b]	49.17[c]	1.806
DMD[++]						
IVDMD (%)[+++]	49.32	49.23	47.14	49.05	52.99	1.365

[abc]Values with different superscript in a row are significantly different (P <0.05).
T1 = 100% forage King grass Ct-115 (Control).
T2 = 99.5% forage King grass Ct-115 plus 0.5% fish oil.
T3 = 95% forage King grass Ct-115 plus 5% fish oil.
T4 = 70% forage King grass Ct-115's 30% *Leucaena leucocephala*.
T5 = 60% forage King grass Ct-115 plus 40% concentrate.
[+] SEM = standard error of the mean.
[++]MSD = Dry matter digestibility.
[+++] IVDMD = *in vitro* digestibility of dry matter expressed as a percentage.

McGeugh *et al*. (2010) mentioned that increasing the starch content in the diet CH_4 formation is reduced without affecting the ruminal metabolism. In contrast, other studies it has been determined that increasing the feed concentrate in the diet, results in acidification of the rumen contents (Cobos *et al.*, 2005), a decrease in the concentration of the cellulolytic bacteria and an increased concentration of amylolytic bacteria; which results in fiber degradation is reduced and the type of fermentation is altered towards the formation of a lower concentration of acetic acid, with an increased production of propionic acid. This coincides with the results obtained in the present study T5, where the pH was lower compared to the other treatments and a slight decrease was observed in the concentration of cellulolytic bacteria, further increase was obtained in the concentration of propionate and lower production of methane.

In conclusion, the addition of 5% fish oil diet for ruminants based on King grass CT-115 pastures, reduces methane production without affecting IVDMD. Expressing CH_4 production depending on the dry matter digestibility of the diet, the same trend was observed. On the other hand, the addition of 0.5% fish oil in the diet had no effect on ruminal fermentation variables and also produces a larger amount of CH_4. Incorporating dietary *L. leucocephala*, had no impact on reducing CH_4 production, and did not affect rumen fermentation variables, not affected population of protozoa. The addition of 5% fish oil and 40% of concentrate feed in diets based on pasture King grass CT-115, decreased CH_4 production, and presented an increase in the concentration of propionate. It is recommended further studies *in vitro* and increases the proportions of fish oil 6–7% to the diet, as well as evaluating diets of lower quality forage such as straw and stubble. It is

suggested that *in vivo* studies to determine the effect of fish oil, *L. leucocephala* and concentrate on the consumption of diet food, weight gain and feed conversion.

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