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Inclusion of sainfoin in beef cattle diet: effects on *in vitro* ruminal parameters.

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INCLUSION OF SAINFOIN IN BEEF CATTLE DIET: EFFECTS ON *IN VITRO* RUMINAL PARAMETERS.

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This work has been accomplished at the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) under the supervision of **Dr. Margalida JOY TORRENS** and **Dr. Sandra LOBÓN ASCASO,**

and publicly defended on September 2023.

INCLUSION OF SAINFOIN IN BEEF CATTLE DIET: EFFECTS ON *IN VITRO* RUMINAL PARAMETERS.

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Abbreviations

ADF…………..Acid detergent fibre

ADL…………..Acid detergent lignin

BH……………Biohydrogenation

BW……………Body weight

C……………….Concentrate

CP…………….Crude protein

CT…………….Condensed tannins

DDM………….Degraded dry matter

DM……………Dry matter

FA…………….Fatty acids

FAME………...Fatty acid methyl esters

GC…………….Gas chromatography

GP…………….Gas production

IVDMD………*In vitro* dry matter degradability

IVOMD………*In vitro* organic matter degradability

ME…………….Metabolizable energy

MUFA………...Monounsaturated fatty acids

NDF…………Neutral detergent fibre

NH3-N………Ammonia

OM……………Organic matter

PUFA…………Polyunsaturated fatty acids

S……………….Straw

SF……………..Sainfoin

SFA…………...Saturated fatty acids

SFH……………Sainfoin hay

SFP……………Sainfoin pellet

UFA…………..Unsaturated fatty acids

VFA…………..Volatile fatty acids

Abstract / Resumen / Résumé

Beef cattle is a strategic livestock activity of great economic, social and environmental interest, as in Spain represents around 15% of the final livestock production and 5% of the value of the final agricultural production. This sector is currently facing different challenges, such as minimizing the environmental impact and avoiding the use of edible food that can be consumed by humans. It is important to promote the circular bioeconomy, using local forage resources and by-products that reduce dependence (especially protein) on imports. One of the forages that stands out is sainfoin, which has a moderate condensed tannins content that can affect ruminal fermentation, depending on its content and chemical structure, diet composition and ruminant species, among other factors. To our best knowledge the use of sainfoin in beef cattle diets for fattening has not been studied.

On this basis, the general objective of this study was to evaluate the inclusion of sainfoin (hay and pellets) in beef cattle diets, on *in vitro* fermentation and ruminal biohydrogenation parameters in two incubation times.

For this purpose, four diets of fattening calves were evaluated on the *in vitro* gas production technique. The diets evaluated were: 90% concentrate and 10% straw (**90C:10S**), 90% concentrate and 10% sainfoin hay (**90C:10SFH**), 85% concentrate and 15% sainfoin pellets (**85C:15SFP**), 70% concentrate and 30% sainfoin pellets (**70C:30SFP**). Three runs were conducted on three separate days and each sample was incubated in triplicate in each run. In addition, eight samples per diet and run were incubated to study biohydrogenation, half of them were collected after 6 h (4 samples/diet/run) and the other half after 24h (4 samples/diet/run).

Our results showed that there was not a clear effect of sainfoin inclusion on gas production. The type of diet had no negative effect on pH, ruminal ammonia concentration, and total production of volatile fatty acids ($P>0.05$). Only the iso-valeric acid was affected by the diet, with lower proportion in the diet with 30% of sainfoin inclusion. In contrast to the effect of type of diet, the incubation time caused a decrease on ammonia and an increase on total volatile fatty acids ($P<0.001$), and in all individual VFA except the acetic acid, which decreased with the time ($P<0.001$).

Regarding the biohydrogenation, the type of the diet induced changes in the fatty acid profile of rumen digesta, with a distinguished rise in C18:3 n-3 as the inclusion of sainfoin was increased. Time was the main factor impact on biohydrogenation, with longer incubation periods altering various fatty acid groups.

Overall, our results support the possibility of including sainfoin in beef cattle diets. However, more research is required to confirm that the inclusion of sainfoin in the diet would not negatively affect beef productivity or product quality. If this is confirmed, the use of sainfoin would contribute to reduce the dependence on imported feed.

El ganado vacuno es una actividad ganadera estratégica de gran interés económico, social y ambiental, ya que en España representa alrededor del 15% de la producción ganadera final y el 5% del valor de la producción agrícola final. Actualmente este sector se enfrenta a diferentes desafíos, como minimizar el impacto ambiental y evitar el uso de alimentos comestibles que podrían ser consumidos por humanos. Es importante promover la bioeconomía circular, utilizando recursos forrajeros locales y subproductos que reduzcan la dependencia (especialmente de proteína) de las importaciones. Uno de los forrajes que se destaca es la esparceta, que tiene un contenido moderado de taninos condensados que pueden afectar la fermentación ruminal, dependiendo de su contenido y estructura química, composición de la dieta y especie de rumiantes, entre otros factores. Hasta donde sabemos, el uso de esparceta no se ha estudiado en dietas de ganado vacuno para engorde.

Con base en esto, el objetivo general de este estudio fue evaluar la inclusión de esparceta (heno y pellets) en dietas de ganado vacuno de carne, sobre los parámetros de fermentación *in vitro* y biohidrogenación ruminal en dos tiempos de incubación.

Para ello se evaluaron cuatro dietas de terneros de engorde mediante la técnica de producción de gas in vitro. Las dietas evaluadas fueron: 90% concentrado y 10% paja **(90C:10S)**, 90% concentrado y 10% heno de esparceta **(90C:10SFH)**, 85% concentrado y 15% pellets de esparceta **(85C:15SFP)**, 70% concentrado y 30% bolitas de esparceta **(70C:30SFP)**. Se realizaron tres experimentos en tres días separados y cada muestra se incubó por triplicado en cada experimento. Además, se incubaron ocho muestras por dieta y tanda para estudiar la biohidrogenación, la mitad de ellas se recogieron después de 6 h (4 muestras/dieta/tanda) y la otra mitad después de 24 h (4 muestras/dieta/tanda).

Nuestros resultados mostraron que no hubo un efecto claro de la inclusión de esparceta en la producción de gas. El tipo de dieta no tuvo efecto negativo sobre el pH, la concentración de amoníaco ruminal o la producción total de ácidos grasos volátiles ($P>0.05$). Sólo el ácido isovalérico fue afectado por la dieta, con menor proporción en la dieta con un 30% de inclusión de esparceta. En contraste con el efecto del tipo de dieta, el tiempo de incubación provocó una disminución en el amoníaco y un aumento en los ácidos grasos volátiles totales ($P<0,001$), y en todos los AGV individuales excepto el ácido acético, que disminuyó con el tiempo ($P<0,001$).

En cuanto a la biohidrogenación, el tipo de dieta indujo cambios en el perfil de ácidos grasos del contenido ruminal, con un marcado aumento en C18:3 n-3 a medida que se incrementó la inclusión de esparceta. El tiempo fue el principal factor de impacto en la biohidrogenación, ya que los períodos de incubación más largos alteraron varios grupos de ácidos grasos.

En general, nuestros resultados respaldan la posibilidad de incluir esparceta en las dietas del ganado vacuno. Sin embargo, se requiere más investigación para confirmar que la inclusión de esparceta en la dieta no afectaría negativamente la productividad de la carne ni la calidad del producto. Si esto se confirma, el uso de esparceta contribuiría a reducir la dependencia de las importaciones.

L'élevage bovin de boucherie est une activité d'élevage stratégique d'un grand intérêt économique, social et environnemental, puisqu'en Espagne il représente environ 15 % de la production animale totale et 5 % de la valeur de la production agricole finale. Ce secteur est actuellement confronté à différents défis, comme minimiser l'impact environnemental et éviter l'utilisation d'aliments comestibles pouvant être consommés par l'homme. Il est important de promouvoir la bioéconomie circulaire, en utilisant des ressources fourragères locales et des sous-produits qui réduisent la dépendance (en particulier les protéines) aux importations. L'un des fourrages les plus remarquables est le sainfoin, dont la teneur modérée en tanins condensés peut affecter la fermentation ruminale, en fonction de son contenu et de sa structure chimique, de sa composition alimentaire et des espèces de ruminants, entre autres facteurs. À notre connaissance, l’utilisation du sainfoin dans l’alimentation des bovins de boucherie destinés à l’engraissement n’a pas été étudiée.

Sur cette base, l’objectif général de cette étude était d'évaluer l'inclusion du sainfoin (foin et granulés) dans l’alimentation des bovins de boucherie, sur les paramètres de fermentation *in vitro* et de biohydrogénation ruminale en deux temps d'incubation.

A cet effet, quatre régimes de veaux en engraissement ont été évalués avec la technique de production de gaz *in vitro*. Les rations évaluées étaient : 90% concentré et 10% paille **(90C:10S)**, 90% concentré et 10% foin de sainfoin **(90C:10SFH)**, 85% concentré et 15% granules de sainfoin **(85C:15SFP)**, 70% concentré et 30% de granules de sainfoin **(70C:30SFP)**. Trois essais ont été effectués sur trois jours distincts et chaque échantillon a été incubé en 3 répliques à chaque essai. De plus, huit échantillons par régime et par essai ont été incubés pour étudier la biohydrogénation, dont la moitié été collectée après 6 h (4 échantillons/régime/essai) et l'autre moitié après 24 h (4 échantillons/régime/essai).

Nos résultats ont montré qu’il n’y avait pas d’effet clair de l’inclusion du sainfoin sur la production de gaz. Le type de régime alimentaire n’a eu aucun effet négatif sur le pH, la concentration d'ammoniac ruminal et la production totale d'acides gras volatils (P > 0,05). Seul l'acide iso-valérique a été affecté par le type de régime alimentaire, avec une proportion plus faible dans le régime avec 30% d’inclusion de sainfoin. Contrairement à l'effet du type de régime alimentaire, le temps d’incubation a provoqué une diminution de l'ammoniac et une augmentation des acides gras volatils totaux (P < 0,001), et de tous

les AGV individuels, à l'exception de l'acide acétique, qui a diminué avec le temps ($P < 0{,}001$).

Concernant la biohydrogénation, le type de régime alimentaire a induit des changements dans le profil des acides gras du digesta du rumen, avec une augmentation notable du C18:3 n-3 à mesure que l'inclusion du sainfoin augmentait. Le temps était le principal facteur d'impact sur la biohydrogénation, des périodes d'incubation plus longues modifiant divers groupes d'acides gras.

Dans l'ensemble, nos résultats soutiennent la possibilité d'inclure le sainfoin dans l'alimentation des bovins de boucherie. Cependant, des recherches supplémentaires sont nécessaires pour confirmer que l'inclusion du sainfoin dans l'alimentation n'aurait pas d'effet négatif sur la production bovine ou sur la qualité du produit. Si cela se confirme, l'utilisation du sainfoin contribuerait à réduire la dépendance vis-à-vis des aliments importés.

Introduction

Beef cattle is a strategic livestock activity of great economic, social and environmental interest. The beef cattle sector in Spain represents around 15% of the final livestock production and 5% of the value of the final agricultural production. Spain has around 6.5 million head of cattle, distributed in approximately 140,000 farms; and contributes to the European Union (EU), around 11% of beef cattle produced (MAPA, 2023). It is the third largest beef producing country, after France and Germany. Within livestock, beef production ranks the third in economic importance, after pork and dairy sector; in fact, in 2022 more than 2.6 million cattle were slaughtered, producing 731.527 tons of meat (MAPA, 2023).

The beef cattle sector is composed by two main sub-sectors: breeding and management of suckling cows and rearing and fattening of beef. The suckler cows are reared under extensive production system, being exposed to important variations of availability and quality of feedstuffs. Farmers feed the cows with their own resources (pastures, rangelands, etc.) and supplement them with straw, preserved fodder and low amount of concentrate, when the pasture is exhausted or not available (mainly winter) (MAPA, 2023). In contrast, beef fattening is reared usually under intensive production system in which animals are kept indoors and fed cereal-based concentrates, with a minimum supply of a low-quality forage (cereal straw). This type of production system started in the 1960s as a consequence of the increase of consumer meat demands, what resulted to a beef intensification evolving to an industrial production system and trying to increase the efficiency, production rate and provide enough meat to the market demand.

However, intensive beef cattle systems entail important negative impacts (Dumont et al., 2014). Therefore, it currently faces different challenges, such as minimizing the environmental impact and avoid the use of edible foods of potential human food consumption. In addition, it is important to promote the circular bioeconomy, using local forage resources and by-products that reduce dependence (especially protein) on imports. In this line, there is a renewed interest in forage legumes because of their important role in sustainable feeding systems due their nitrogen fixation ability and their potential high nutritional value for ruminants. Based on the above-mentioned challenges, one of the resources that stands out in the Mediterranean area, is the Sainfoin (*Onobrychis viciifolia*) with a high nutritional value and high digestibility coefficient for ruminants. This forage has a moderate condensed tannins content (Mueller-Harvey et al., 2019), which can affect ruminal fermentation depending on its content and chemical structure, diet composition and ruminant species, among other factors. However, in our knowledge, the use of sainfoin in beef cattle fattening diets has not been studied.

Bibliographic review

Ruminants are characterized by having a complex system of stomachs that allows prolonged contact between ingested feed and a rich flora of microorganisms in order to favour the fermentation of the former and its better use. In fact, ruminal microorganisms are able of degrading the structural carbohydrates of plant cell walls, making them into energy available to ruminants (Jarrige et al., 1995). Therefore, we should take advantage of this capacity and encourage a greater use of forages in the ruminants' diet, decreasing the use of cereals and imported feeds. In addition, in recent years, there has been a growing interest in the use of local forage resources, which responds to the need for greater self-sufficiency, efficiency and sustainability on the part of the farms. There is especial interest in leguminous crops because of their soil restoring action and their high nutritional value to feed ruminants. In the current study, we study the inclusion of sainfoin in diets for beef cattle, which is explained in more detail below.

1. Sainfoin

Sainfoin is a traditional forage legume, with a high palatability, nutritive value with high crude protein content (Delgado et al., 2008a). Sainfoin is grown in dry hilly environments on calcareous soils and it originated from South Central Asia (Mora-Ortiz & Smith, 2018). During the last decades in Europe, more noticed in France, Italy and Spain, the cultivation of sainfoin had declined steadily until nowadays, mainly because its low persistence and low regrowth after the first spring cut (Borreani et al., 2003). Besides, currently there is a wide availability of forage crop varieties, great interest of farmers using intensive production systems (Carbonero et al., 2011; Demdoum et al., 2012), and low-cost nitrogen fertilizers avoiding the need of intercrop leguminous (Carbonero et al., 2011), what helped decrease their cultivation. Other reasons why the use of sainfoin declined, was the rise of feed imports from non-European Union (EU) countries, and this has led to increasing dependence of Europe on import of animal feed (Galloway et al., 2008).

The annual distribution of sainfoin production was studied in Aragón, under rainfed and irrigated conditions. The results obtained showed a similar distribution of annual forage production in both conditions, being 66.9%, 21.4% and 11.7% in the first, second and third cuts under rainfed conditions, and 67.2%, 19.4%, 9.9% and 3.5% in the first, second, third and fourth cuts under irrigated conditions, respectively (Delgado et al., 2008b). The usual form of use of sainfoin is to perform a spring mowing for hay, in full bloom, and a cut or grazing of the regrowth in autumn or winter, although it is also ensiled (Cavallarin et al., 2005; Delgado et al., 2008a). Thus, as the production at the first cut is about the 2/3 of total production, the sainfoin should be preserved in form of hay or silage to be used during the shortage periods (Carbonero et al., 2011).

The sainfoin has a high digestibility (Aufrère et al., 2008; Theodoridou et al., 2010). Its CP content is high (up to 200 g/kg DM) and its average NDF content is 450 g/kg DM (Delgado et al., 2010). The quality of this forage can vary according to the variety, the phenological state and the conservation method (Hatew et al., 2015; Sheppard et al., 2019; Wang et al., 2015). Besides, the interest in sainfoin forage has increased due to its average of condensed tannins (CT) content at a concentration of 10-90 g/kg DM (Mueller-Harvey et al., 2019). This type of secondary compounds has shown beneficial effects for animal production, such as a reduced risk of bloat (McMahon et al., 1999), a decrease in methane, gas and ammonia production (Hatew et al., 2016), a decrease in protein degradation as evidenced by decreased ammonia and isoacid contents (Rufino-Moya et al., 2020), as well as a concomitant increase in protein flow to the small intestine (Kraiem et al., 1990), a reduction on the impact of intestinal nematodes (Hoste et al., 2015) and a beneficial modification of ruminal biohydrogenation (BH) capable of improving the fatty acid (FA) profile of the final product (Frutos et al., 2020). The condensed tannins can modulate the ruminal lipid metabolism, because they affect negatively to some species of fibrolytic bacteria and ciliate protozoa, what may inhibit some steps of the BH of FA (Vasta et al., 2007).

Although the abovementioned effects are accepted as overall, there is some controversy about the extent of them. Some studies found no effect in gas and methane production, ammonia content or total VFA production (Grosse Brinkhaus et al., 2017; Guglielmelli et al., 2011; Theodoridou et al., 2010), whereas in other studies it was observed reduction in *in vitro* digestibility (Niderkorn et al., 2012), gas (Calabrò et al., 2012; Niderkorn et al., 2012; Theodoridou et al., 2011), methane production and protein digestion (Waghorn, 2008). The reduction of protein digestion may only be beneficial when the protein in the diet is higher than requirements but not when it is insufficient (Mueller-Harvey et al., 2019).

2. *In vitro* gas production technique

Nutritive value of a ruminant feed is determined by its chemical composition, as well as their rate and extent of digestion. To do that, it should study the digestibility of the diet, through *in vivo*, *in sacco* or *in vitro* assays. The studies *in vivo* can reproduce the usual conditions of animal production, but it is laborious, expensive, requires large quantity of feed and time, and is largely unsuitable for single feedstuffs. As alternative, the *in vitro* methods are more attractive because they are less expensive, and easier to carry out, able to assess a large number of samples at the same time, and need a smaller number of animals (Getachew et al., 2005). Besides to the abovementioned characteristics, on *in vitro* methods the incubation conditions can be well controlled (Getachew et al., 2002). *In vitro* gas production is a laboratory technique that allow to study the production of gas and methane recorded during the process of ruminal microbial fermentation. It simulates rumen conditions, analysing the amount of gas produced and the correlation with methane (Menke et al., 1979).

In vitro gas production consists of incubating feed with ruminal fluid plus buffer solution under anaerobic conditions at constant temperature of 39°C to reproduce the ruminal environment. This technique facilitates the study of the incidence of individual factors as diet (Tang et al., 2008), type of inoculum (Calabrò et al., 2005), presence of secondary compounds (Makkar, 2005) on fermentation parameters, and predict the nutritional value of the feed (Williams, 2000). Therefore, *in vitro* ruminal fermentation is used to evaluate ruminal fermentation processes, such as gas (GP), ammonia (NH_3-N), methane (CH_4) and volatile fatty acids (VFA) production (Blümmel et al., 2005; Getachew et al., 2005). Due to the wide variability among individual rumen microbiome, it has been recommended that *in vitro* assay should include multiple runs, at least 3 runs in 3 different days. The differences in ruminal content are associated to the variations in animal rumen volume, passage rates as well as the individual characteristics of digestive process. Besides the microbial populations can vary the activities in the starting inoculum or in the survival or activity of microbes over the incubation period, which is another reason why there should be multiple runs incubated (Yáñez-Ruiz et al., 2016).

On the other side it should be taken into consideration that *in vitro* gas production technique only generates an approximation of what happens in *in vivo* conditions, since it lacks the complexity that normally occurs in the rumen of the animal and therefore cannot be a strictly precise indication of the interactions between the components present in the feed and the microbial population.

3. *In vitro* fermentation products

In vitro gas technique primarily measures the digestion of soluble and insoluble carbohydrates (Menke and Steingass, 1988). When a substrate is fermented *in vitro* under the above-mentioned conditions with buffered rumen fluid, it produces gases (mainly CO_2 and CH_4), VFA and NH_3-N (Getachew et al., 1998).

3.1. Carbon dioxide and methane

The amount of gas produced from a ruminal feed fermentation reflects production of CO_2, CH_4 and volatile fatty acids (VFA), which are the major source of energy for ruminants. Gas arises directly from microbial degradation of feeds, and indirectly from buffering of acids generated as a result of fermentation (Getachew et al., 2004). Compared to carbohydrate fermentation, gas production from protein fermentation is relatively small (Wolin, 1960).

Enteric methane emitted by ruminants is one of main concerns related to livestock activity as it is the most abundant greenhouse gases (GHG) emitted at the farm scale in ruminant production systems. The CH_4 is produced as a byproduct of the digestive process and represents a significant energy loss from the animal, ranging from 2–12% of gross energy intake (Martin et al., 2010). Thus, enteric CH_4 production concerns arise due to the environmental issue and the associated loss of energy for the animal. Once ingested, feedstuff is broken into monomers by ruminal microorganisms, which later anaerobically ferment them producing VFA, mainly acetate, propionate, and butyrate, and CO_2, hydrogen (H_2), CH_4 (Nagaraja, 2016). The CH_4 production is not constant and is influenced by several factors, being the most determinant the presence of structural carbohydrates. Diets rich in fibre increase CH_4 production, while diets based on cereal-rich feeds reduce it (Rooke et al., 2014).

The interest in ruminant production is the reduction of ruminal CH_4 emissions because it will involve an increase of the efficiency of fermentation, and more energy will be able for animal production (Martin et al., 2010). For this reason, recently feeding studies had been focussed on looking for strategies to reduce enteric CH_4 production.

Several strategies have been studied to mitigate these emissions, among which highlights the presence of condensed tannins (CT) in the diet of ruminants (Puchala et al., 2005). Condensed tannins can reduce CH_4 production through a direct effect on rumen methanogenic bacteria and a decrease in the number of symbiotic protozoa, as well as through an indirect effect on the production of hydrogen, due to a lower degradation of the feed (Tavendale et al., 2005).

3.2. Volatile Fatty Acids

The nutrients required for maintenance and production of ruminants are obtained from the digestive processes of feed fermentation by bacteria and protozoa, with volatile fatty acids as the final product, which also are transformed to glucose, amino acids or fatty acids by rumen bacteria.

The main VFA produced in the rumen are acetic, propionic, butiric, iso-butyric, iso-valeric and valeric acids. The three first VFA accounts for the largest proportion, whereas the rest are present in smaller amount. The VFA plays a vital role in ruminant nutrition and are crucial indicators of rumen fermentation. They are the most important contribution to the energy of the ruminants, which represents 65-75% of the total metabolizable energy (Bergman, 1990). Carbohydrates are the main source of VFA production, from which it is mainly generated acetic, propionic and butyric acids (Getachew et al., 1998).The protein contributes in a minor way to the production of VFA, through the degradation of the carbon structure of the amino acids during the fermentation (Van Soest, 1994), especially in these diets that the content of protein is high. Iso-butyric, iso-valeric and valeric acids are produced by the degradation of the valine, leucine and proline amino acids, respectively (Zavaleta, 2002).

The type of diet plays a determinant role in the production of the three main VFA (acetic, propionic and butyric). Acetic fermentation predominates in fodder rations, while in concentrated based diets, propionic acid fraction will increase in detriment of acetic (Cheeke & Dierenfeld, 2010). During fermentation, VFA can undergo a mutual conversion process, which can be explained seeing that a certain VFA is the end product of the activity of some microorganisms, which in turn, is the substrate for other microorganisms. So, we can observe that between 40 and 80% of butyric acid derives from acetic acid and 6 to 20% of acetic acid comes from butyric acid (R. Weston & Hogan, 1968). The molar proportions of VFA, point out which type of fermentation pattern had followed the substrates, mainly acetic or propionic fermentations, so that the acetate: propionate ratio has been widely used as a parameter to evaluate the type of fermentation (Getachew et al., 1998). The type of diet determines the fermentation pattern and gas production. Under acetic fermentations it is promoted great gas production, while under propionic fermentation happens the contrary, it produces less gas (Getachew et al., 1998; Wolin, 1960). When the fermentation is propionic there is a reduction of the acetate: propionate ratio, and also there is a reduction of the free H_2 ion, the major substrate to produce CH_4 for methanogenic bacteria (Beauchemin et al., 2009; Carulla et al., 2005). The total VFA produced, 75% of them will be absorbed directly in the reticulum-rumen, while the rest will be absorbed in the omasum and abomasum, and as little as 5 % will reach the intestine. After the absorption process VFA reaches to the tissues, where they are oxidised to provide energy for the animal, or to the liver, where they are used for the synthesis of fat (acetic and butyric acids) or glucose (propionic acid) (Bondi, 1988).

3.3. Ammonia

Ruminal ammonia represents a critical aspect of *in vitro* fermentation, as its presence is of paramount importance as it serves as a vital source of nitrogen for microbial protein synthesis (Dijkstra et al., 2018), except that some rumen bacteria can use small peptides and amino acids for their growth, (McDonald et al., 2010). Proteins present in forages are usually degraded, producing ammonia as a byproduct of amino acid deamination (McMahon et al., 2000).

The ruminal content of ammonia can be reduced with the presence of condensed tannins, because they bond to the proteins, reducing their degradation in the rumen (Mueller-Harvey, I. 2006). Rufino-Moya et al. (2021) in a review exposed that the presence of CT in the diet has been associated with a reduction in NH_3-N content because they reduce the solubility and degradation of dietary proteins in the rumen. In addition, the presence of CT causes a slower fermentation rate that allows a better synchronization between nitrogen and energy, which reduces the NH_3-N content and allows a higher microbial protein synthesis (Mueller-Harvey, I. 2006). This effect is clearly visible in the *in vitro* tests carried out with fresh sainfoin at different incubation times (Niderkorn et al., 2012; Rufino-Moya et al., 2019; Theodoridou et al., 2011). In contrast, Chung et al. (2013) did not find any difference in the ruminal content of ammonia, neither when was fresh or hay sainfoin. However, the studies regarding the effect of CT on the fermentation parameters are inconclusive, what can be related to the type and concentration of CT and to the content of crude protein of the diet.

4. Biohydrogenation

Consumers are increasingly concerned about the nutritional quality of food. Health professionals have recommended increasing the intake of PUFA in diets (especially long-chain n-3 PUFAs) and reducing the ratio of PUFA n-6: n-3. Therefore, there is growing interest in identifying strategies to enhance the healthy FA in food as a proportion of total lipids (Chikwanha et al., 2018). It is known that the ruminal biohydrogenation process affects the fatty acid profile to be depot in animal tissues, therefore it is essential to know these ruminal processes, in order to obtain the meat and milk quality demanded by consumers.

Most of the fatty acids of the forage are unsaturated FA (UFA), and the ones from the rumen are mostly saturated fatty acids (SFA). The lipids that enter in the rumen are transformed by two major processes, lipolysis and BH, which convert UFA to SFA. When the lipids enter in the rumen, first it happens the lipolysis process, in which the microbial lipases hydrolyse the ester linkages in complex lipids, which causes the release of FA (Buccioni et al., 2012). After the process of lipolysis is finished, those unsaturated FA undergo BH by ruminal microbes. Rumen BH is a very complex process with a series of steps and pathways. The UFA are generally saturated through the ruminal population as a mechanism of detoxication in response to the inhibitory effect of UFA on microbial growth (Jenkins et al., 2008) being the C18:0 the last product of the BH. Consequently, the final FA profile of ruminant-derived products will be more saturated FA due ruminal BH (Scollan et al., 2017). The process of lipolysis and BH depends on the type and amount of fat that comes through the diet, and also on the ruminal pH (Jenkins et al., 2008). In addition to the BH process, rumen bacteria are also able to synthesize odd and branched chain FA, which have been suggested as potential biomarkers of rumen function (Vlaeminck et al., 2015). As Frutos et al. (2020) reported in their review, FA are divided in four main origin groups: dietary PUFA, intermediate metabolites of rumen, bacterial FA and products of de novo synthesis. They also pointed that: the more is known about the benefits of PUFA on human health, more will be the interest in ruminant researchers to find ways to protect the dietary UFA from ruminal BH, that will lead to the increase of their concentration in the products for human consuming. Nevertheless, during the BH process there are intermediate stages forming intermediate FA, some of which are unique in ruminant products and beneficial for consumers health, such as some PUFA n-3, CLA c9, t11 and C18:1 t11 (Buccioni et al., 2015; Shingfield et al., 2008). Since ruminal BH is never produced to the same extent, the degree to which it is completed determines whether the final profile is more or less rich in PUFA, intermediate FA or saturated FA, being this process of relevance for consumers health (Shingfield et al., 2008).

The FA profile of animal products depends on numerous factors, such as the age, weight, breed, sex and feeding system (Chikwanha et al., 2018). However, the main factor affecting the FA profile is the feeding system (Raes et al., 2004), therefore the ingredients of the diet play an important role in the research of strategies to modify the FA profile. The increase of forage proportion and the inclusion of condensed tannins in the diet have been studied to improve the FA profile of animal products, as they are able to modify the biohydrogenation in the rumen, reducing the ruminal saturation of dietary FA and at the same time increasing the concentration of PUFA and some intermediate FA with beneficial properties (Frutos et al., 2020). In addition, forages have a very beneficial FA profile, as they are mostly composed of PUFA, being C18:3 n-3 the major FA (Glasser et al., 2008), making them an interesting strategy to improve the FA profile of meat and milk.

Objectives

The present Mater Thesis was proposed with the general objective to evaluate the inclusion of sainfoin in beef cattle diets, on *in vitro* fermentation and ruminal biohydrogenation parameters.

To address this general objective, the following partial objectives were proposed:

1. Evaluate the inclusion of sainfoin, in different preservation methods (hay and pellets) in beef cattle diets on *in vitro* fermentation and biohydrogenation parameters.
2. Evaluate the inclusion of sainfoin in beef cattle diets at different incubation time *in vitro* fermentation and biohydrogenation parameters.

Material and methods

1. Diets, animals, experimental design and sampling

All procedures were carried out in accordance with the Spanish guidelines for experimental animal protection (RD 53/2013).

Four diets of fattening calves were evaluated on the *in vitro* study in the CITA Research Centre in Zaragoza (41◦3′ N, 0◦47′ W, Spain). Commercial concentrate, straw, sainfoin hay and sainfoin pellets were used as ingredients. Four diets were evaluated: 90% concentrate and 10% straw (**90C:10S**), 90% concentrate and 10% sainfoin hay (**90C:10SFH**), 85% concentrate and 15% sainfoin pellets (**85C:15SFP**), 70% concentrate and 30% sainfoin pellets (**70C:30SFP**).

Six cows (697 ±23.8 kg BW), used as donors of rumen inoculum, were fed commercial concentrate (5kg/day) plus straw, water and mineral blocks *ad libitum*. After 4 weeks receiving this diet for adaptation, and in three consecutive weeks, rumen liquid was collected through an oesophageal tube, discarding the first part of the extraction to avoid possible contamination with saliva. To avoid the excessive manipulation of the animals, 2 animals were used each week. Ruminal liquid was obtained always before the morning feeding into a pre-warmed (39°C) insulated thermos, with the intention to keep it at constant temperature, and it was immediately transported to the laboratory, which was located next to the animal facilities. The ruminal liquid was strained through four layers of cheesecloth, mixed and homogenized. A buffer solution was added based on the protocol of Menke and Steingass (1988) in a proportion of 1:2 (v/v) (rumen fluid: buffered solution, v/v) under a continuous flow of CO_2, presenting a pH of 6.8 ±0.01.

All the incubations were carried out using bottles of 310 mL, equipped with pressure and temperature sensors of Ankom® system (Ankom Technology, Macedon NY, USA). In each bottle 500 mg of the diet and 60 mL of mixed rumen fluid: buffered solution was added. The bottles were hermetically sealed under anaerobic conditions, in the presence of CO_2 and placed in a 39°C bath. Three runs were conducted on three separate days and each sample was incubated in triplicate in each run. Three bottles without substrate were used as negative controls (blanks). Gas production was recorded for 24 h.

During this 24 h incubation time, the gas production was recorded hourly by the sensor present in the head of the Ankom System bottle. When the incubation time was over, the bottles were placed in ice for 5-10 min to stop fermentation and then tempered at room temperature for 10-15 min. After this, the pH was measured with a microPH 2002 (Crison Instruments S.A, Barcelona, Spain). The entire bottle content was filtered through a preweighed bag (50 µm; Ankom) to estimate the *in vitro* dry matter degradability (IVDMD) and the *in vitro* organic matter degradability (IVOMD). The bags with samples were sealed and washed twice with distilled water and were dried at 103 °C for 48 h. After 48 h, the bag content was weighed and IVDMD was calculated. The dried sample was placed in a muffle at 550 °C to obtain the ashes. The organic matter of bag content was obtained as DM-ashes, and the IVOMD was calculated.

To study the biohydrogenation, 8 samples per diet and run were incubated, half of them were collected after 6 h (4 samples/diet/run) and the other half after 24 h (4 samples/diet/run), following the same procedure as described previously for gas production, the samples were placed in ice for 5-10 minutes and then tempered at room temperature for 10-15 min. In order to have 2 samples/diet and run, the contents of two bottles were mixed and kept as one sample. Immediately the samples were frozen at -80 °C. Then, samples were freeze-dried and stored at - 80 °C until FA analysis.

Samples of the liquid, from 6 and 24 h, were taken to determine ammonia (NH3-N) and volatile fatty acids (VFA). In order to determine NH3-N, 2.5 mL of liquid was mixed with 2.5 mL HCl 0.1 N, and to determine VFA, 0.5 mL of the liquid was added to 0.5 mL of deproteinizing solution (containing 4-methyl-valeric acid as internal standard) and 1 mL of distilled water and they were stored at -20 °C until those analysis were realized.

2. Chemical analysis

2.1. Feedstuffs

The chemical composition analyses of the feed samples (commercial concentrate, straw, sainfoin hay, sainfoin pellet and the 4 composed diets) were analysed at the laboratory of Nutritive Value of CITA. All the chemical analyses were run in duplicate.

The contents of Dry matter (DM) (index no. 934.01), ash (index no. 942.05), were determined according to the AOAC methods (AOAC, 2000). The DM content was determined by drying in a Model ULM 400 furnace (Memmert, Schwabach, Germany) with forced ventilation at 103 °C until constant weight was reached (method 934.01). Afterwards, the dry sample was calcined in a Model 367.PE furnace- muffle (P-Selecta, Barcelona, Spain) at 550 °C for 6 hours to determine the ash content (942.05 method). Organic matter (OM) was determined as the difference of dry matter and ashes. Crude protein (CP) content was determined by Dumas Procedure (968.06 method) using a Model NA 2100 (Thermoquest SA, Barcelona, Spain).

Neutral detergent fiber (NDFom), acid detergent fiber (ADFom), and acid detergent lignin (lignin (sa)) contents were determined according to the method described by Van Soest et al. (1991) using the Ankom 200/220 fibre analyser (Ankom Technology Corporation, Fairport, NY, USA). The NDFom from forages was assayed with a heat stable amylase (amylase and at 80 °C for 1 h). The lignin (sa) was analysed on ADFom residues by solubilization of cellulose with sulphuric acid. All values were corrected for ash-free content. The total starch of the diets was measured with the commercial kit K-TSTA-100A (Neogen Corporation, Lansing, MI, USA) following the amyloglucosidase/α-amylase method. The estimation of Metabolizable Energy of the concentrate was calculate following the FEDNA tables (FEDNA, 2019), considering the proportion of each ingredient included in the concentrate. The ME of Straw, SF Hay and SF Pellet was determined based on the equations of Mertens, (1993). The chemical composition of the ingredients is shown in Table 1.

Table 1. Chemical composition of the ingredients used in the diets.

	Ingredients			
Item	Concentrate	Straw	SF Hay	SF Pellet
Ash, g/kg DM	56.3	99.8	65.6	81.3
CP[1], g/kg DM	154.8	37.2	-	138.3
NDFom[2], g/kg DM	178.9	775.1	402.9	434.1
ADFom[3], g/kg DM	66.2	438.2	276.3	287.4
ADL[4], g/kg DM	8.3	41.7	58.5	57.4
Crude fat, g/kg DM	38.6	-	-	-
ME[5], MJ/kg DM	11.6	6.4	10.6	10.4

[1]crude protein. [2]neutral detergent fibre exclusive of residual ash. [3]acid detergent fibre exclusive of residual ash. [4]acid detergent lignin. [5]metabolizable energy.

2.2. Condensed tannins determination

Determination and characterization of total condensed tannis was done as Extractable Condensed Tannins (ETC), Protein Bound Condensed Tannins (PBCT) and Fibre Bounded Condensed Tannis (FBCT) based on (Terrill et al., 1992) and quantified at 550 nm based on the method described by (Grabber et al., 2013). The standards used for quantification of the samples were extracted and purified based on the method described (Wolfe et al., 2008).

2.3. Volatile Fatty Acids determination

Volatile Fatty Acids in ruminal liquid were determined by gas chromatography (GC) in (Jouany, 1982). The concentrations of acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids were determined using a Bruker Scion 460 GC (Bruker, USA) GC equipped with CP- 8400 autosampler, a DB-SWax capillary column (30 m × 0.25 mm i.d × 0,250.25 µm film thickness, Agilent, USA), a flame ionisation detector and controlled by a Compass CDS software. The injection volume was 1 µl at a split ratio of 1:50. Helium was used as a carrier gas with a flow of 1 mL/min. The oven temperature program was 110°C, followed by a 6°C/min increase to 158°C and held for two minutes. The VFA were identified based on retention time comparisons with commercially available standards of acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and 4-methyl-valeric acids (≥ 99% purity, Sigma-Aldrich, USA) and quantified with an external calibration curve using 4-methyl- valeric as internal standard.

2.4. Ammonia-N determination

Ammonia-N content in rumen was determined by a colorimetric method based on Berthelot reaction (Chaney & Marbach, 1962), where ammonium chloride was used as standard. In order to do ammonia-N determination, the samples and the standards were treated with phenol and sodium hypochlorite, then left in the incubation for 45 min at room temperature and finally were measured at 625 nm in Epoch spectrometer plate (BioTek Instruments, Inc., Winooski, VT, USA).

2.5. Fatty Acids determination

The concentrations of FA in feeds and rumen were determined as FA methyl esters (FAMEs) by GC. Samples of 500 milligrams of freeze-dried were bimethylated with $NaOCH_3$ and acetyl chloride, extracted with heptane following the indications of Lee et al. 2012. Later, they were injected into a Bruker Scion 460 GC (Bruker,USA) GC equipped with CP-8400 autosampler, a HP-88 capillary column (100 m × 0.25 mm i.d × 0.20 μm film thickness, Agilent, USA), a flame ionisation detector and controlled by a Compass CDS software. The injection volume was 1 μl at a split ratio of 1:15. Helium was used as a carrier gas with a flow of 1.5 mL/min. The oven temperature program was 125°C held for 15 minutes, followed by a 5°C/min increase to 165°C, which was held for 45 minutes, then by a 5°C/min increase at 190°C, held for 50 minutes and in the end a 5°C/min increase at 200°C and held for 48 minutes. The FAMEs were identified based on retention time comparisons with commercially available standards: GLC-532, GLC-401, GLC-643, GLC-642, GLC-463, C18:1 c11, C18:1 t11, C19:0, standard references (Nu-Chek- Prep Inc., Elysian, Minnesota, USA), Mixture BR, Mixture BR 4 (Larodan, Solna, Sweden) and the relative retention times observed in the bibliography (Alves & Bessa, 2009; Kramer et al., 1997). FAMEs quantification was performed as described in (ISO 12966-4, 2015) expressed as a percentage of the total amount of the total identified FAMEs or as mg FAMEs/g freeze-dried samples using C19:0 as internal standard.

The calculations corresponding to the biohydrogenation estimates (%) in rumen are described in Alves et al. (2017). The C18:1 c9, C18:2n−6 and C18:3n−3 biohydrogenation were obtained from the variation between the diet and rumen respect to the proportion in diet. The calculations of biohydrogenation are:

$$\text{Maximum C18: }0_{\text{Rumen}} = (\text{C18: 1 c}9_{\text{Diet}} - \text{C18: 1 c}9_{\text{Rumen}}) + (\text{C18: 1 c}11_{\text{Diet}} - \text{C18: 1 c}11_{\text{Rumen}}) + (\text{C18: 2n} - 6_{\text{Diet}} - \text{C18: 2n} - 6_{\text{Rumen}}) + (\text{C18: 3n} - 3_{\text{Diet}} - \text{C18: 3n} - 3_{\text{Rumen}}) + \text{C18: }0_{\text{Diet}}$$

where C18:0 in rumen, is the C18:0 in rumen as percentage of total C18 FA; C18:1 $c9_{\text{Diet/Rumen}}$: is the C18:1 c9 in Diet or Rumen as percentage of total C18 FA; C18:1 $c11_{\text{Diet/Rumen}}$: is the C18:1 c11 in Diet or Rumen as percentage of total C18 FA; C18:2n−$6_{\text{Diet/Rumen}}$: is the C18:2n−6 in Diet or Rumen as percentage of total C18 FA;

$C18{:}3n{-}3_{Diet/Rumen}$: is the C18:3n−3 in Diet or Rumen as percentage of total C18 FA; $C18{:}0_{Diet}$: is the 18:0 in Diet as percentage of total C18 FA (Alves et al., 2017).

3. Statistical analyses

All the data from this trial were analysed using the statistical software SAS (v. 9.4; SAS Inst. Inc., Cary, NC; EE. UU).

Fermentation parameters were analysed through a general linear model (GLM) with the diets and run as fixed effect. The least square means and their standard error means (SEM) were obtained. Volatile fatty acids (VFA), fatty acid methyl ester (FAMEs), and biohydrogenation % were also analysed with a GLM with diets, incubation time (hours), run and the interaction of diets with incubation time as fixed effects. The least square means and their standard error of means (SEM) were obtained. The effects were considered significant at $P<0.05$.

Results and discussion

1. Chemical composition

Table 2 shows the chemical composition of the four diets studied (70C:30SFP; 85C:15SFP; 90C:10SFH and 90C:10S) and their fatty acid profile. The chemical composition of the diets changed as forage was included in the diet. The inclusion of sainfoin pellet increased the fibre fractions and decreased the starch content. All diets have similar condensed tannins contents, which was low (1.8 to 2.1 g eq. CT sainfoin/ kg DM). High content of condensed tannins can be considered detrimental for ruminants (Mueller-Harvey, 2006), but low to moderate content of them can be beneficial (Frutos et al., 2020). However, the minimum level of condensed tannins that may affect the animal performance has not yet been established. The metabolizable energy ranged from 11.5 MJ/kg DM in the 90C:10SFH to 11.1 MJ/kg DM in 90C:10S diets, being the narrow variation due to the differences on the chemical composition.

Regarding the FA profile, the main FA of the diets were C16:0, C18:1 c9 and C18:2n-6, which could have been affected by the conservation method. It was observed that the inclusion of sainfoin caused an increase of C18:3n-3 in the same sense of the level of inclusion. The proportion of PUFA was similar among diets, and the MUFA was numerically greater in the diet with straw, while SFA was numerically greater in the diet 70C:30SFP.

2. Ruminal fermentation

In vitro gas production is an important indicator to assess rumen fermentation status, which can reflect the fermentative activities of rumen microorganisms and the extent of diet degradation (Cattani et al., 2014). The results obtained of the *in vitro* fermentation trial are shown in Table 3. The initial ruminal pH was 6.81 and at the end of study all diets presented a similar final pH, with an average of 6.47 ± 0.02 ($P>0.05$), indicating that the conditions of fermentation were adequate for the study, without acidosis. The addition of a ratio of 2:1 buffer solution: ruminal liquid allowed to develop a fermentation with similar conditions to the animal, respect to the pH values, which is supported by Rotger et al. (2006), as they found similar final pH with the present study, when they evaluated concentrates *in vitro* and *in vivo* in cattle.

Table 2. Chemical composition of the diets and their fatty acids (FA).

Chemical composition	Diets[1] 70:30 C: SFP	85:15 C: SFP	90:10 C: SFH	90:10 C: S	SE[2]
Ash, g/kg DM	56.6	56.9	55.1	55.3	0.45
CP[3], g/kg DM	147.7	147.6	151.6	140.4	2.34
NDFom[4], g/kg DM	264.6	233.8	210.5	236.9	11.08
ADFom[5], g/kg DM	132.2	102.7	88.9	98	9.37
ADL[6], g/kg DM	29.3	22.9	22.3	22.1	1.73
Crude fat, g/kg DM	32.9	34	36.8	34.4	0.82
Starch, g/kg DM	295.1	383.3	412.3	416.3	28.19
ME[7] MJ/kg DM	11.2	11.4	11.5	11.1	0.10
Total condensed tannins (CT)[8]	1.93	1.83	2.14	1.89	0.067
TOTAL FA (mg FAMEs/g DM)	53.34	60.2	65.97	62.31	2.7
FA profile %					
C12:0	3.03	3.03	3.05	3.00	0.01
C14:0	1.59	1.59	1.56	1.64	0.02
C16:0	32.67	31.3	31.23	31.12	0.4
C18:0	7.6	6.88	6.85	6.82	0.2
C18:1 c9	29.26	30.49	31.04	31.2	0.4
C18:1 c11	0.82	0.84	0.85	0.86	0.0
C18:2n-6	22.51	23.81	23.95	24.25	0.4
C18:3n-3	2.54	2.06	1.46	1.1	0.3
Sums[9]					
SFA	44.88	42.8	42.69	42.59	0.5
MUFA	30.07	31.33	31.89	32.06	0.5
PUFA	25.05	25.87	25.42	25.34	0.2

[1]C: commercial concentrate; SFP: sainfoin pellet; SFH: sainfoin hay; S: straw. [2]standard error. [3]crude Protein. [4]Neutral Detergent Fibre. [5]Acid Detergent fibre. [6]Acid Detergent Lignin. [7]Metabolizable energy. [8]g eq. CT sainfoin/kg DM. [9]SFA: Saturated fatty acids; MUFA: Mono-unsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

Improving microbial efficiency, while maximizing feed conversion into microbial biomass, improving microbial protein supply to the small intestine and reducing energy losses is one of the main objectives of nutritional researchers (Beever et al., 1993). In the present study, the diet affected the total gas production (mL/g incubated DM/OM; $P<0.05$), being lower in the diet 70C:30SFP than in 85C:15SFP ($P<0.05$), meanwhile the diets 90C:10SFH and 90C:10S presented intermediate values. However, when the total gas production was related to the DM or OM degraded (mL/degraded DM), the diet did not affect this parameter (P>0.05). Regarding the IVDMD and IVOMD, both were affected by the type of the diet (P<0.001), showing greater digestibility the diets constituted by 85C:15SFP and 90C:10SFH compared to 90C:10S, and 70C:30SFP (P<0.05). These results showed that the lower gas production observed in 70C:30SFP and 90C:10S is related to a lower ruminal degradation recorded in these diets, whereas the highest gas production is registered in the diets with higher rate of in vitro digestibility. The high IVDMD and IVOMD in 85C:15SFP and 90C:10SFH diets with respect to the rest of diets might be an indication that the fermentation of DM or OM led to increases in the microbial biomass, as total VFA production and NH_3-N content were similar among diets.

Table 3. Effect of the type of diet on gas production, in vitro dry matter degradability (IVDMD), in vitro organic matter degradability (IVOMD) at 24h.

Item	Diets[1]					
	70:30 C: SFP	85:15 C: SFP	90:10 C: SFH	90:10 C: S	SEM[2]	*P*-value
Final pH	6.51	6.45	6.47	6.45	0.02	0.2925
Total GP[3], mL/g iDM[4]	138^{b}	146^{a}	145^{ab}	139^{ab}	1.6	0.0229
Total GP, mL/g iOM[5]	131^{b}	138^{a}	137^{ab}	133^{ab}	1.5	0.0392
Total GP, mL/g dDM[6]	166	171	170	171	1.7	0.2206
Total GP, mL/g dOM[7]	156	159	158	160	1.8	0.3776
IVDMD, %	82.91^{b}	85.41^{a}	85.16^{a}	81.35^{b}	0.35	0.0005
IVOMD, %	84.04^{b}	86.95^{a}	86.68^{a}	82.96^{b}	0.28	0.0001

[1]C: commercial concentrate; SFH: sainfoin hay; S: straw; SFP: sainfoin pellet. [2]standard error of the mean. [3]gas production. [4]iDM: incubated dry matter. [5]iOM: incubated organic matter. [6]dDM: degraded dry matter. [7]dOM: degraded organic matter. The different lowercase letters indicate differences among diets at $P < 0.05$.

The contents of NH_3-N and VFA are shown in Table 4. No interaction between diet and incubation time was observed (P>0.05), thus the effects are presented separately. The diet did not affect VFA or NH_3-N studied (P>0.05), except for iso-valeric acid (P<0.001), which was higher in both diets with 90% of concentrate (90C:10SFH, and 90C:10S) than in the diets with inclusion of sainfoin pellet. The similar total VFA production is consistent with the similar chemical composition of the diets. In general, the presence of structural carbohydrates is linked with the production of acetic and butyric acids to the detriment of propionic acid (Calabrò et al., 2005). In the present study, no effect of the inclusion of forage was observed in acetic and propionic acids, indicating that the proportion of forage was not enough to change the type of fermentation. The greater content of iso-valeric acid in the both diets with 90% of concentrate does not reflect the CP content of the diets as it is found in (Guglielmelli et al., 2011), suggesting that the diets might have changed the ruminal microbiota, which involves the formation of many branched-chain amino acids, probably through the incorporation of propionyl-CoA into the carbon skeletons (Van Soest, 1994). So, when the branched-chain amino acids (BCAA) valine, leucine, and isoleucine are oxidatively deaminated, they are converted to the branched-chain fatty acids (BCFA) isobutyric, isovaleric and 2-methylbutyric acid, respectively (Apajalahti et al., 2019). Thus, suggesting that there was enough availability of branched-chain amino acids, which are necessary for iso-fatty acids synthesis.

Table 4. Effect of diets on ammonia (NH3-N) and volatile fatty acids (VFA).

	Diets[1]					
Item	70:30 C: SFP	85:15 C: SFP	90:10 C: SFH	90:10 C: S	SEM[2]	*P*-value
NH_3-N mg/L	26.2	29.9	30.7	32.0	3.25	0.64
Total VFA, mmol/L	47.8	49.0	47.9	47.9	1.61	0.94
Molar proportion, %						
Acetate	75.7	75.1	74.1	74.7	1.10	0.76
Propionate	15.5	15.8	16.5	16.0	1.10	0.94
Isobutyrate	0.45	0.46	0.43	0.45	0.024	0.92
Butyrate	7.2	7.5	7.8	7.7	0.24	0.42
Isovalerate	0.57^{b}	0.63^{ab}	0.69^{a}	0.66^{a}	0.016	<.0001
Valerate	0.54	0.53	0.54	0.50	0.021	0.56
Acetate: Propionate ratio	5.2	5.2	5.0	5.1	0.33	0.96

[1]C: commercial concentrate; SFH: sainfoin hay; S: straw; SFP: sainfoin pellet. [2]standard error of the mean. The different lowercase letters indicate differences among diets at $P < 0.05$.

The effect of time was significant in all the parameters studied (P<0.05). At 6 h of incubation, the content of NH_3-N was greater than at 24 h (Figure 1), whereas the total VFA (Figure 1) and almost all the individual VFA (Figure 2) were lower. The ammonia produced in the rumen serves as a nitrogen source for rumen microbes for their own growth and reproduction, producing microbial protein as a result. Based on the results of the present study, we suggest that ammonia contributes indirectly to the increase to VFA production as the microbial population and its metabolism increased. Acetic acid ($P<0.05$; Figure 2), was the only individual VFA which presented higher values at 6 h than at 24h, this might be because this VFA is accomplished largely in the rumen when there is low carbohydrates availability (Van Soest, 1994). Also, VFA can undergo an interconversion process during fermentation, which can be explained by taking into account that a certain VFA are the end product of the activity of some microorganisms and, in turn, are the substrate for other microorganisms. Thus, we can observe that between 40 and 80% of butyric acid derives from acetic acid and 6 to 20% of acetic acid comes from butyric acid (R. H. . Weston & Hogan, 1968). Then suggesting that acetic acid is consumed by several microorganisms as a source of energy and giving other VFA as final product. The acetate: propionate ratio was almost twice at 6 h than 24 h ($P<0.05$; Figure 3), this is due to the amount of acetic acid at 6 hours compared to 24 hours, as the acetic acid is abundantly larger than propionic acid, which in 24 hours increased.

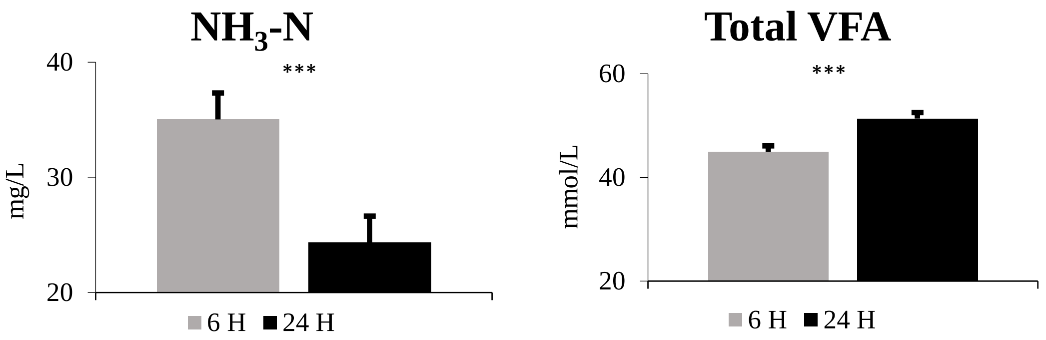

Figure 1. NH3-N content and total VFA at 6 and 24 h of incubation.

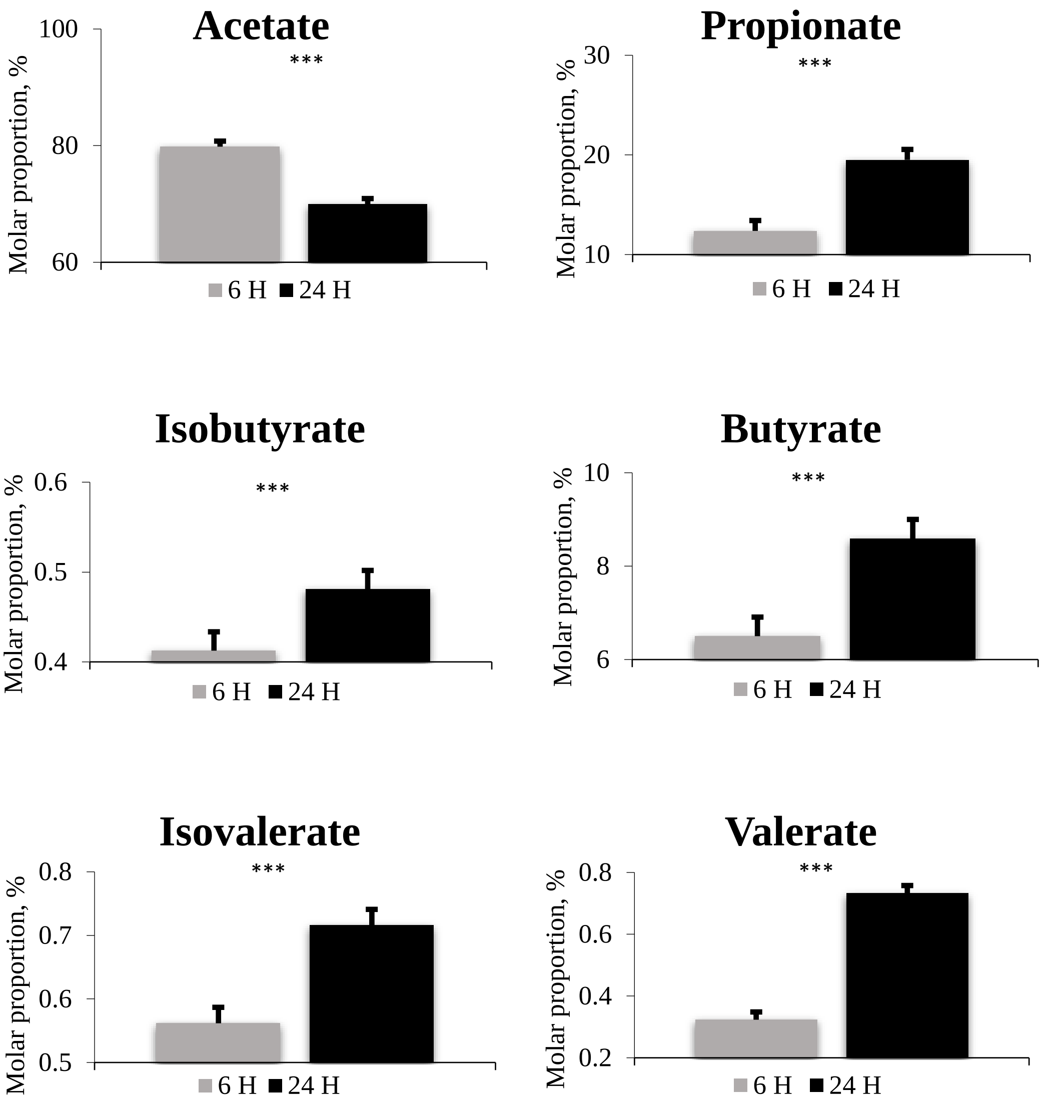

Figure 2. Individual proportions of VFA at 6 and 24 h of incubation.

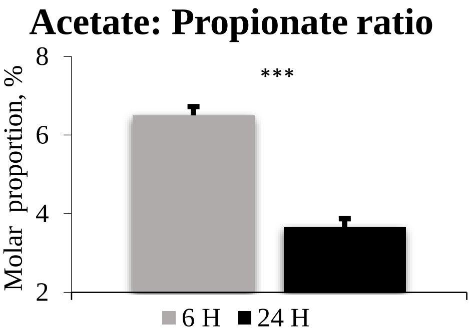

Figure 3. Acetate: Propionate ratio at 6 and 24 h of incubation.

For all the Figures: ***P-value <0.001.

3. Ruminal Biohydrogenation

The inclusion of sainfoin pellets and hay in the diets might have played an important role in the changes occurred in the *in vitro* ruminal BH. The significant effects on the ruminal FA showed that the diets evaluated had an effect on the *in vitro* ruminal BH. Table 5 reports the FA profile (% total FA), SFA and BCFA of *in vitro* ruminal liquid for the 4 diets and at two incubation times. No interaction between diet and incubation time was found (P>0.05), except for C20:0 and C22:0 (P<0.05). At 24h of incubation all diets presented higher proportions of both FA, but this increase was only significant in the diets 70C:30SFP (P<0.05). The increase of both FA according to the diets and time of incubation is drawn in Figure 4 and 5, for C20:0 and C22:0, respectively.

The total FA (mg FA/g DM), was not affected by the type of diet and the incubation time (P>0.05), even though there was a numerically decrease at 24h of incubation. Total SFA percentage was affected by type of diet (P<0.01), with higher proportion in the diets 70C:30SFP and 90C:10S compared to 90C:10SFH (P<0.05), while 85C:15SFP presented intermediate values (P>0.05). Regarding the individual proportions of SFA, the diet only had effect on C17:0, C21:0 and C24:0 (P<0.05). Most of them were greater in the 70C:30SFP than the rest of diets (P<0.05), except the C17:0 which presented intermedium proportions in C85:15SFP and C90:10SFH (P>0.05) and C21:0 which was similar to C85:15SFP.

The incubation time also affected the total SFA proportion (P<0.001), being higher at 24 h (P<0.05), suggesting that as the incubation time increased, the biohydrogenation of the unsaturated FA also increases. Most of the individual SFA proportion were affected by the incubation time (P<0.001), recording the highest proportion at 24 h except for C12:0, C18:0, C21:0, C23:0, C24:0, C26:0 and C28.0, which were unaffected by the incubation time (P>0.05), what could be related to the speed at which there are saturated. There is a noticeable increase of C18:0 percentage at 6 and 24 h of incubation with respect to this FA in diets.

The presence of condensed tannins of sainfoin in the diet would modify the biohydrogenation of FA (Vasta et al., 2009). However, in the present study, after sainfoin pelletization, the presence of these secondary compounds was minimal, so their effect on ruminal biohydrogenation is expected to be negligible. Toral et al. (2016) studying alfalfa hay vs. sainfoin hay in *in vitro* biohydrogenation, observed that the expected inhibitory effect of tannins on ruminal BH, a reduction of C18:0 accumulation in the digesta, was just recorded numerically no statistically, and only differences were seen at 6h of incubation.

There were identified seven branched chain fatty acids, including *iso* and *anteiso*. The BCFA are formed from the action of rumen bacteria on branched-chain amino acids (valine, leucine and isoleucine) and branched-short-chain carboxylic acids (isobutyric, isovaleric and 2-methyl butyric acids) (Fievez et al., 2012). Their profile in the rumen seems to be determined by the fatty acid synthase activity of the microorganisms, so these BCFA reflect changes in the specific bacterial populations in the rumen, which are related to the diet (Vlaeminck et al., 2006). Any of BCFA studied were affected by diet ($P>0.05$), suggesting that the diets were not sufficiently different between them to have important effects on the ruminal microbiota composition, or conceivably, it might be needed to adapt the ruminal liquid to the diet, prior the *in vitro* incubation, to find the changes in the BCFA. Regarding to the incubation time, it was observed that all BCFA were affected by the incubation time ($P<0.05$), being higher at 24 h compared to 6 h in all proportions. This increase with incubation time was expected, as there is an increase of the activity of microbiota (Toral et al., 2016).

Table 5. Effect of diets and incubation time on ruminal SFA and BCFA.

Item	Diets[1]					Incubation time			*P*-value	
	70:30 C: SFP	85:15 C: SFP	90:10 C: SFH	90:10 C: S	SEM[2]	6H	24H	SEM	Diet	I. time
Total FA, mg FA/g DM	15.5	15.7	19.0	21.2	1.59	19.3	16.4	1.13	0.07	0.10
Individual FA, %										
SFA [3]	60^{a}	58^{ab}	58^{b}	61^{a}	0.7	56	62	0.5	0.007	<.0001
C12:0	1.57	1.74	1.71	1.70	0.054	1.73	1.63	0.038	0.15	0.09
C13:0	0.12	0.11	0.10	0.10	0.012	0.04	0.17	0.009	0.43	<.0001
C14:0	3.09	2.90	2.76	2.92	0.092	2.15	3.69	0.065	0.14	<.0001
C15:0	1.03	0.95	0.89	0.94	0.106	0.39	1.52	0.075	0.85	<.0001
C16:0	32	32	32	32	0.3	31	33	0.2	0.95	<.0001
C17:0	0.34^{a}	0.31^{ab}	0.30^{ab}	0.29^{b}	0.012	0.25	0.37	0.008	0.03	<.0001
C18:0	20.6	18.6	18.6	21.7	0.95	19.2	20.6	0.67	0.09	0.16
C20:0*	0.86^{a}	0.66^{b}	0.56^{bc}	0.50^{c}	0.033	0.56	0.73	0.023	<.0001	0.0001
C21:0	0.03^{a}	0.03^{a}	0.02^{b}	0.02^{b}	0.002	0.02	0.02	0.001	0.03	1
C22:0*	0.24^{a}	0.19^{b}	0.18^{b}	0.17^{b}	0.007	0.17	0.21	0.005	<.0001	<.0001
C23:0	0.05	0.04	0.04	0.03	0.004	0.04	0.04	0.003	0.06	1
C24:0	0.20^{a}	0.17^{b}	0.17^{b}	0.15^{b}	0.006	0.16	0.18	0.005	0.002	0.09
C26:0	0.09	0.10	0.09	0.08	0.022	0.09	0.09	0.016	0.96	0.80
C28:0	0.42	0.51	0.33	0.37	0.112	0.38	0.43	0.080	0.67	0.71
BCFA [4]	2.2	2.0	1.7	1.8	0.16	1.0	2.9	0.11	0.17	<.0001
iso-BCFA[5]	1.19	1.07	0.93	0.97	0.086	0.52	1.56	0.061	0.17	<.0001
anteiso-BCFA[6]	1.04	0.94	0.82	0.85	0.079	0.51	1.31	0.056	0.24	<.0001

[1]C: Commercial concentrate; SFH: Sainfoin hay; S: Straw; SFP: Sainfoin pellet. [2]standard error of the mean. [3]Total Saturated FA. [4]Total Branched Chain FA. [5]sum of individual iso-C13:0, iso-C14:0, iso-C15:0, iso-C16:0, iso-C17:0. [6]sum of individual anteiso-C15:0, anteiso-C17:0. *Significant in the interaction between diets and incubation time, $P<0.05$. The different lowercase letters indicate differences among diets at $P < 0.05$.

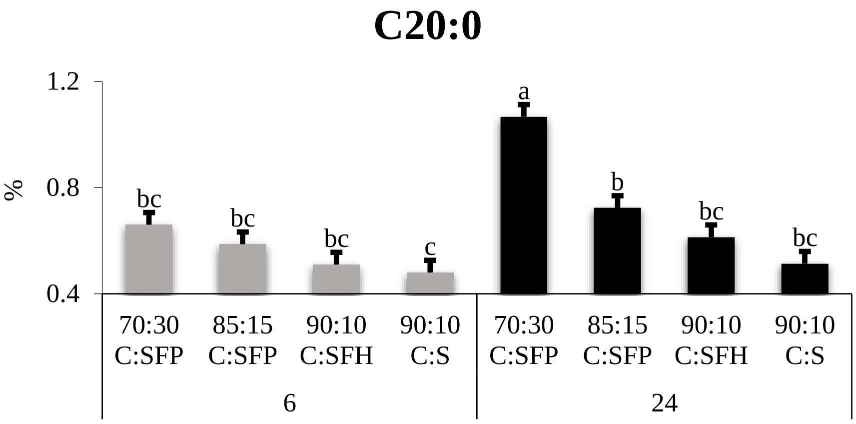

Figure 4. Interaction between diet and incubation time on C20:0.

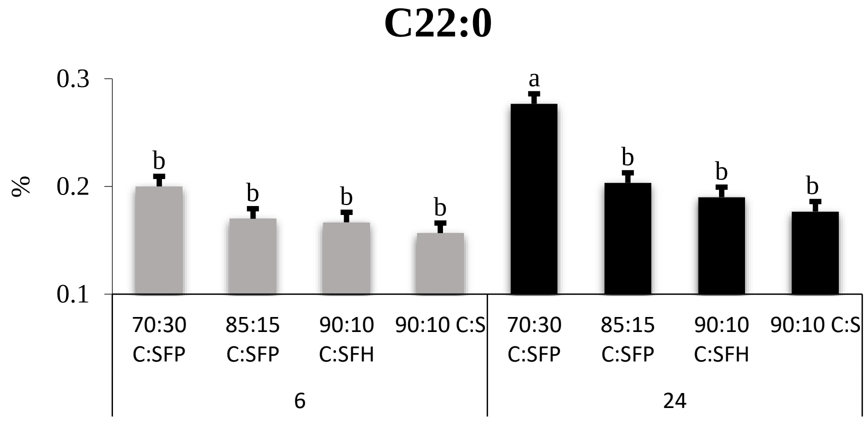

Figure 5. Interaction between diet and incubation time on C22:0.

Table 6 represents the effect of diets and incubation time on ruminal MUFA of the *in vitro* assay. Only C18:1 c16 presented interaction between diet and incubation time ($P<0.05$; Figure 6), showing all diets greater percentage at 24 h than 6 h of incubation ($P<0.05$), except the diet 90C:10S ($P>0.05$). This increase between both times was more marked in the diets 85C:15SFP and 90C:10SFH, as at 6 h their percentages were low, whereas at 24 h were high. The change in the diet 70C:30SFP, was less noticeable than the rest of diets.

There were identified sixteen individual MUFA. The effect of diet was significant in total MUFA ($P<0.01$), total cis-MUFA ($P<0.001$), C16:1c7 ($P<0.001$), and C18:1c9 ($P<0.05$). The diet 90C:10SFH showed greater proportion of total MUFA, total cis-MUFA and C18:1 c9 than the 70C:30SFP ($P<0.05$), while 85C:15SFP and 90C:10S had intermediate values (P>0.05). Regarding the oleic acid (C18:1 c9), its proportion in the ruminal liquid comes from the diet and/or from the biohydrogenation. In the present *in vitro* study, the greater C18:1 c9 percentage observed in 90C:10SFH cannot be related to its content in the diet, because all diets presented similar percentages among them. Therefore, the differences could be related with the biohydrogenation of C18:2 n-6 (Honkanen et al., 2012). In contrast the C16:1c7 FA presented greater proportion in the diet 70C:30SFP that the rest of diets (P<0.05).

Incubation time had effect on total MUFA (P<0.001), total cis-MUFA (P<0.001), and total trans-MUFA (P<0.05), and to the individuals FA C16:1 c7, C16:1 c9, C18:1 c9, C18:1 c11 (P<0.001), C18:1 t5 (P<0.05), C18:1 t9 (P<0.001), C18:1 t11 (P<0.01). At 6 h total MUFA, total cis-MUFA and C18:1 c9, presented greater proportion than at 24 h, while the rest of MUFA presented greater proportion at 24h of incubation. The lower proportion of C18:1 c9 at 24h is a result of the biohydrogenation, what is in concordance with the observations of Huyen et al. (2020) and (Toral et al., 2016).

Some of the effects produced by the inclusion of sainfoin pellet and hay can be observed on *trans*-MUFA group during the incubation time. The mayor *trans*-FA produced during ruminal BH are C18:1 t10 and C18:1 t11. The accumulation of C18:1 t10 in the rumen liquid at the expense of C18:1 t11 is known as “t10-shift” whose occurrence is related to concentrate-rich diets (Griinari et al., 1998). It is desirable that C18:1 t11 increases and C18:1 t10 decreases, due to their potential effects on human health (Palmquist et al., 2004). Concerning the C18:1 t10, whose potential specific role for human health and animal performance is still unclear (Shingfield et al., 2008), but no variations were detected among the diets and time. Nevertheless, a great individual variability in t10-shift has been reported in animals fed the same diet (Bessa et al., 2015). The C18:1 t11 proportion was unaffected by the diet, but did increase with incubation time, what is desirable because it is able to be converted into CLA c9, t11 by Δ^9-desaturation of C18:1 t11 in the body tissues, having both FA a favourable effect on the human heath (Palmquist et al., 2005).

Table 6. Effect of diet and incubation time on ruminal MUFA.

	Diets[1]					Incubation time			*P*-value	
Item	70:30 C: SFP	85:15 C: SFP	90:10 C: SFH	90:10 C: S	SEM[2]	6H	24H	SEM	Diet	I. time
MUFA [3]	23^{b}	25^{ab}	26^{a}	25^{ab}	0.4	26	23	0.3	0.003	<.0001
cis-MUFA	17.2^{b}	19.2^{a}	20.19^{a}	18.1^{ab}	0.43	21.3	15.9	0.3	0.001	<.0001
C16:1 c7 [4]	0.26^{a}	0.19^{b}	0.18^{b}	0.16^{b}	0.009	0.16	0.23	0.006	<.0001	<.0001
C16:1 c9	0.4	0.52	0.58	0.36	0.084	0.15	0.77	0.06	0.27	<.0001
C18:1 c9	14.77^{b}	16.63^{ab}	17.52^{a}	15.92^{ab}	0.572	19.9	12.52	0.405	0.03	<.0001
C18:1 c11	1.36	1.51	1.59	1.25	0.194	0.8	2.06	0.137	0.61	<.0001
C18:1 c12	0.16	0.14	0.14	0.14	0.008	0.14	0.15	0.006	0.28	0.27
C18:1 13c	0.02	0.02	0.02	0.02	0.002	0.02	0.02	0.001	0.13	0.67
C18:1 c14 [5]	0.15	0.13	0.13	0.17	0.014	0.15	0.14	0.01	0.27	0.20
C18:1 c15	0.01	0.01	0.01	0.01	0.002	0.01	0.01	0.001	0.39	0.43
C18:1 c16*	0.03	0.03	0.03	0.04	0.002	0.03	0.04	0.001	0.54	0.0001
trans-MUFA	5.7	5.3	5.5	6.6	0.62	5	6.6	0.44	0.50	0.02
C18:1 5t	0.06	0.07	0.04	0.06	0.014	0.04	0.07	0.01	0.65	0.048
C18:1 t6/t7/t8 [6]	0.44	0.41	0.46	0.54	0.055	0.41	0.51	0.039	0.43	0.10
C18:1 t9	0.37	0.37	0.38	0.44	0.029	0.31	0.47	0.02	0.29	<.0001
C18:1 t10	0.45	0.42	0.45	0.55	0.051	0.44	0.5	0.036	0.32	0.27
C18:1 t11	3.9	3.6	3.73	4.48	0.453	3.24	4.62	0.32	0.55	0.01
C18:1 t12	0.29	0.27	0.28	0.33	0.027	0.31	0.28	0.019	0.45	0.24
C18:1 t15	0.18	0.2	0.2	0.22	0.021	0.21	0.18	0.015	0.66	0.20

[1] C: commercial concentrate; SFH: sainfoin hay; S: straw; SFP: sainfoin pellet. [2] standard error of the mean. [3]total monounsaturated FA. [4]C16:1 c7 might coelute with C16:1 t3. [5]C18:1 t16 coelutes with C18:1 c14 as minor isomer. [6]C18:1 t6, C18:1 t7, and C18:1 t8 might coelute. *Significant in the interaction between diets and incubation time, P<0.05. The different lowercase letters indicate differences among diets at $P < 0.05$.

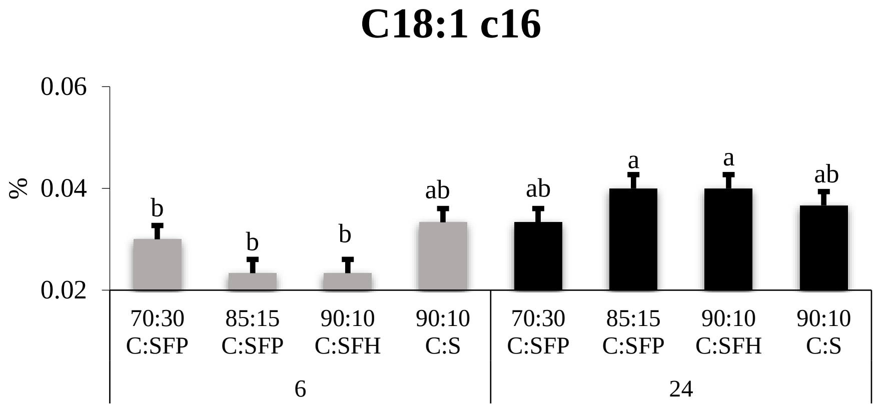

Figure 6. Interaction between diet and incubation time on C18:1 c16.

Table 7 shows the effect of diets and incubation time on ruminal PUFA of the *in vitro* assay. No interaction was observed between diet and incubation time (P>0.05); thus, the effects are presented separately. The type of diet only had effect on C18:2 t10, c15 (P<0.05), C18:3 n-3 (P<0.001), and C18:0 oxo-12/-13 (P<0.05). The diet 70C:30SFP presented the greatest proportion percentage of C18:3 n-3, whereas the 90C:10S behaved inversely (P<0.05). The C18:2 t10, c15 proportion was higher in 70C:30SFP than in 90C:10SFH, while the rest presented intermediate values. The C18:0 oxo-12/-13 proportion was higher in 90C:10S than in 70C:30SFP, while the rest presented intermedium values. The FA composition of the ruminal digesta in the diets with sainfoin pellets and hay, particularly the C18:3n-3, suggests that the diets with sainfoin could affect the ruminal BH as the presence of this FA was lower in the diet 90C:10S (P<0.05), what is in agreement with the results observed by Toral et al. (2016). This former effect was more marked in the diet 70C:30SFP, what might be related to the greater proportion of this FA in the diet.

Incubation time had significant effect on most of the individual PUFA (P<0.05), except for the C18:2 t10, c15 and C18:3 n-3 (P>0.05), what could be due to a low biohydrogenation in low amounts of sainfoin, making changes hard to detect (Mueller-Harvey, I. 2006) At 6 h of incubation, total PUFA, C18:2 n-6, CLA, CLA c9, t11; CLA t10, c12 had greater proportion than at 24 h, which might be related with the capacity that the bacteria have to hydrogenate dietary unsaturated fatty acids, leading to a reduction in the proportion of unsaturated fatty acids over the time (Jenkins et al., 2008). Meanwhile C18:3 c9, t11, c15, oxo-FA, C18:0 oxo-12/-13, C18:0 oxo-10 presented greater proportion at 24 h, might indicate a shift in microbial populations that might preferentially produce these fatty acids (Patra & Saxena, 2011).

Table 7. Effect of diets and incubation time on ruminal PUFA and oxo-FA.

Item	Diets[1]					Incubation time			*P*-value	
	70:30 C: SFP	85:15 C: SFP	90:10 C: SFH	90:10 C: S	SEM[2]	6H	24H	SEM	Diet	I. time
PUFA [3]	11	12	12	10	0.7	14	8	0.5	0.15	<.0001
C18:2 n-6	8.6	10	10.2	8.4	0.67	12.4	6.2	0.48	0.17	<.0001
C18:2 t10, c15 [4]	0.1^{a}	0.06^{ab}	0.05^{b}	0.06^{ab}	0.011	0.07	0.06	0.008	0.04	0.16
C18:3 n-3	1.39^{a}	0.99^{b}	0.83^{b}	0.43^{c}	0.087	0.95	0.87	0.061	<.0001	0.35
C18:3 c9, t11, c15	0.13	0.13	0.09	0.08	0.021	0.08	0.13	0.015	0.27	0.048
CLA [5]	0.53	0.52	0.59	0.56	0.033	0.69	0.41	0.023	0.42	<.0001
CLA c9, t11	0.42	0.41	0.44	0.41	0.019	0.54	0.3	0.013	0.65	<.0001
CLA t10, c12	0.12	0.11	0.15	0.15	0.02	0.15	0.1	0.014	0.33	0.02
oxo-FA	0.24	0.26	0.4	0.45	0.057	0.15	0.53	0.041	0.05	<.0001
C18:0 oxo-12/-13 [6]	0.16^{b}	0.18^{ab}	0.23^{ab}	0.30^{a}	0.032	0.07	0.36	0.023	0.04	<.0001
C18:0 oxo-10	0.07	0.08	0.17	0.16	0.034	0.08	0.17	0.024	0.14	0.02

[1]C: commercial concentrate; SFH: sainfoin hay; S: straw; SFP: sainfoin pellet. [2]standard error of the mean. [3]total polyunsaturated FA. [4]C18:2 t10, c15 might coelute with C18:2 t11, c15. [5]total conjugated linoleic acid. [6]C18:0 oxo-12 and C18:0 oxo-13 might coelute. *Significant in the interaction between diets and incubation time, P<0.05. The different lowercase letters indicate differences among diets at P < 0.05.

Several minor intermediates FA from BH process were detected in rumen liquid, particularly CLA isomers or oxo-18:0. During this *in vitro* incubation it was favoured the production of CLA c9, t11 and CLA t10, c12 at 6 h of incubation, whereas decreased at 24 h, regardless of the type of diet. Vasta et al. (2009) suggested that tannin supplementation could be a good strategy to increase CLA c9, t11. However, in the present study the CT content in the diets was too low to observe any effect. The concentration of C18:0 oxo-10, which is a metabolite that results from a pathway of hydration and oxidation of UFA from the diet (Jenkins et al., 2008), was not different among diets, contrary to Toral et al. (2016), who found lower concentration of C18:0, oxo-10 in sainfoin *in vitro* incubations. This result confirms that the diets of the present study had low presence and activity of CT to observe the expected effects of sainfoin.

The estimated C18 biohydrogenation of unsaturated FA in ruminal liquid are shown in Table 8. The diets had no effect on biohydrogenation extent for C18:1 c9, C18:2 n-6 ($P>0.05$), what is related to the lack of effect of the CT. Only a tendency was observed in C18:3 n-3 ($P=0.07$) with a greater BH extent in 90C:10S than the rest of diet with presence of sainfoin. Huyen et al. (2020), studying the inclusion of sainfoin silage in diet of cows found a lower extent of biohydrogenation cis-9-C18:1and C18:3n-3, concluding that sainfoin decreased the ruminal C18:3n-3 biohydrogenation, even though the low presence of CT. (Vasta et al., 2009) in an *in vitro* study reported that tannins reduced ruminal biohydrogenation by the inhibition of ruminal microorganism rather than by a direct interaction of tannins with the enzymes involved in the biohydrogenation pathway. In addition, (Sackmann et al., 2003) reported that the process of biohydrogenation increases at higher rates when the forage content in the diet is increased, which can be related to greater presence of the microorganisms involved in the BH, mainly cellulolytic bacteria (Kepler & Tove, 1967). Also, we might not have seen effect, because the donor animals of the rumen liquid from the current study were adapted to the same diet: concentrate (kg/d) and straw *ad libitum*. While C18:3 n-3 showed that 90C:10S has a tendency to present a higher biohydrogenation than 75C:30SFP and 90C:10SFH, while 85C:15SFP showed intermediate values ($P<0.1$), which might be due to the activity of tannis, although the quantity of tannins is not very high.

In our study, the incubation time had effect on C18:1 c9 and C18:2 n-6 ($P<0.001$) BH extent, being higher at 24 h.

Table 8. Biohydrogenation extent, %.

Item	Diets[1]					Incubation time			P-value	
	70:30 C: SFP	85:15 C: SFP	90:10 C: SFH	90:10 C: S	SEM[2]	6H	24H	SEM	Diet	I. time
BH extent, %										
C18:1 c9	49.5	45.5	43.6	49.0	1.86	34.8	59.0	1.32	0.1191	<.0001
C18:2 n-6	61.8	57.8	57.6	65.5	2.82	47.6	73.7	1.99	0.1989	<.0001
C18:3 n-3	45.5	52.0	43.2	61.5	4.77	47.3	53.8	3.38	0.0703	0.1949

[1] C: commercial concentrate; SFH: sainfoin hay; S: straw; SFP: sainfoin pellet. [2] standard error of the mean.

Conclusions

Under the conditions of the present study, it can be concluded:

1. The diet had effect on the gas production related to the dry matter incubated, however this effect disappeared when it was expressed related to the dry matter degraded.
2. The inclusion of 15% of sainfoin pellets or 10% of sainfoin hay in the diet increased *in vitro* degradability, whereas greater percentage of sainfoin in the diet caused lower degradability.
3. Ruminal fermentation parameters, such as ammonia and total volatile fatty acids production, were not affected by the diet. Increasing the time of incubation causes a decrease of ammonia and an increase of total volatile fatty acids.
4. Diet had effect on the fatty acid profile of ruminal content. The percentage of C18:3 n-3, was increased as the percentage of forage increased in the diet. Furthermore, other effects on total cis-MUFA and individual FA as oleic acid (C18:1c9) were observed. In contrast, CLA and total PUFA percentages were not affected by diet.
5. The biohydrogenation extent was not affected by the diet, although 90C:10S had a tendency to present higher C18:3 n-3 biohydrogenation.
6. In the present study the time was the major factor affecting the biohydrogenation. Most of the groups of FA, were affected by the time of incubation, increasing the SFA and trans-MUFA, whereas the MUFA and cis-MUFA, and PUFA decreased with the time.
7. Further *in vivo* research is recommended to investigate the possible effects of sainfoin inclusion in the diet of beef cattle on performance and meat quality.

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