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INTERNATIONAL MASTER OF ANIMAL NUTRITION

Mealworm (*Tenebrio molitor*) oil as an alternative to vegetable fats: Effect on ruminal fermentation and biohydrogenation.

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MEALWORM (*TENEBRIO MOLITOR*) OIL AS AN ALTERNATIVE TO VEGETABLE FATS: EFFECT ON RUMINAL FERMENTATION AND BIOHYDROGENATION.

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This work has been accomplished at the *Instituto de Ganadería de Montaña* (CSIC-University of León) under the supervision of **Drs. Clàudia BAILA BIGNÉ and Pilar DE FRUTOS FERNÁNDEZ**

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ABBREVIATIONS

A/P .....Acetate:propionate ratio

ADF.....Acid detergent fibre

ADL.....Acid detergent lignin

BCFA.....Branched-chain fatty acids

BH .....Biohydrogenation

CLA .....Conjugated linoleic acid

CO .....Canola oil

CP .....Crude protein

DM .....Dry matter

DMD.....Dry matter disappearance

DMI .....Dry matter intake

EE .....Ether extract

EU.....European Union

FA .....Fatty acid

FAME.....Fatty acid methyl esters

FM .....Fresh matter

FO.....Fish oil

GC.....Gas chromatography

ivTSD .....*In vitro* true substrate digestibility

LA .....Linoleic acid

LNA.....Linolenic acid

MUFA .....Monounsaturated fatty acids

NDF .....Neutral detergent fibre

NDFD.....Neutral detergent fibre disappearance

OCFA .....Odd-chain fatty acids

OM .....Organic matter

PFA .....Palm distillate fatty acids

PO .....Palm oil

PUFA .....Polyunsaturated fatty acids

RA.....Rumenic acid

SA .....Stearic acid

SBO .....Soybean oil

SED.....Standard error of the difference

SFA .....Saturated fatty acids

SFO.....Sunflower oil

TM.....*Tenebrio molitor*

TMO .....*Tenebrio molitor* oil

TMR.....Total mixed ration

UFA .....Unsaturated fatty acids

VA.....Vaccenic acid

VFA.....Volatile fatty acids

SUMMARY

Climate change, geopolitical instability, and population growth have intensified the feed-food-fuel competition. Add to this the consumer demand for healthier products obtained under environmentally friendly conditions and it is easy to understand why the search for alternative sources for livestock feeding has become a global priority. One of these alternative sources can certainly be insects.

Ruminant diets are generally supplemented with lipids to enhance their energy content and enhance animal performance and/or to modulate ruminal biohydrogenation and increase the content in meat and milk of potentially healthy fatty acids (FA) for consumers. Some of the most commonly used fats in ruminant diets are those derived from the oil palm plant and the soybean oil (SBO), but they both have a high environmental impact. *Tenebrio molitor* oil (TMO), an insect-derived lipid with an unsaturated lipid profile, could be an alternative lipid source for ruminant feeding.

This MSc thesis was carried out with the aim of investigating the effects of dietary supplementation with *T. molitor* oil on ruminal fermentation, degradation and biohydrogenation processes in sheep, using three substrates with different F:C ratios to simulate different feeding systems. These effects were compared to those of diet supplementation with either palm distillate fatty acids (PFA) or SBO.

To this aim, an *in vitro* study was conducted using batch cultures of rumen microorganisms and the gas production technique. Three rumen cannulated ewes were used as donors of the inoculum. Three substrates diverging in F:C ratio were prepared: 100:0 (called 100% F), 50:50 (called 50:50 F:C), and 10:90 (called 10:90 F:C). Each of them was supplemented with each of the following lipid sources at 2% DM: PFA, SBO and TMO.

The incubations lasted for 3 different times depending on the incubated substrates: 8 h for the 10:90 F:C, 20 h for the 50:50 F:C, and 30 h for the 100% F. Then, we analysed some parameters as indicative of rumen fermentation and degradation (e.g., gas production, ammonia and lactic acid concentration, volatile fatty acid production, estimated CH_4 and CO_2 production, and disappearance of DM and NDF, and *in vitro* true substrate digestibility) and biohydrogenation (rumen digesta FA composition).

Our results showed that TMO hardly affected *in vitro* ruminal fermentation and degradation across the 3 substrates studied. If differences were observed when comparing the inclusion of the 3 lipid sources (i.e., PFA, SBO, and TMO), they were minor.

The effects of TMO on ruminal BH showed a little more variation depending on the basal diet. However, several consistent patterns were identified across the 3 diets studied.

Higher proportions of odd-chain FA, which are associated with potential health benefits in humans, were detected in the rumen digesta of substrates supplemented with TMO compared to PFA. Results were similar for TMO and SBO.

Most of the differences observed were attributed to the specific fatty acid composition of PFA, SBO and TMO. These effects seemed to happen: i) directly, through the transfer from the diet to the rumen digesta (e.g., 16:0 with PFA, *cis*-9 18:1 and 14:0 with TMO, and *cis*-9 *cis*-12 18:2 with SBO), or ii) indirectly, via the BH of unsaturated FA contributed by the supplements (e.g., higher concentrations of some intermediates, such as certain conjugated linoleic acid (CLA) isomers or *trans*-monounsaturated FA, with SBO and TMO, and of 10-oxo-18:0 with TMO).

Supplementation with *T. molitor* oil increased *trans*-11 18:1 concentration compared to PFA. However, it also led to higher levels of *trans*-10 18:1 in the 100% F and 50:50 F:C substrates. Notably, no shift in the *trans*-10/*trans*-11 ratio was observed in any case. Patterns similar to those of *trans*-11 18:1 were found for *cis*-9 *trans*-11 CLA in the same substrates (100% F and 50:50). These results were comparable or slightly less favourable than those obtained with SBO. However, in some cases, SBO caused higher increases of *trans*-10 18:1 and *trans*-10 *cis*-12 CLA, both potentially detrimental for dairy ruminants.

Overall, our findings support the use of *T. molitor* oil to replace PFA and SBO in sheep, to alleviate not only the competition for limited resources but also the harmful impact of these conventional sources on the environment. However, further studies are required to confirm our results and to investigate the potential of TMO on animal performance and product (meat and milk) quality, as well as other aspects related, for example, to the extrapolation of these results to different production contexts.

RESUMEN

El cambio climático, la inestabilidad geopolítica y el crecimiento de la población han intensificado la competencia entre la producción de alimentos para humanos, para animales y para biocombustibles (*feed-food-fuel*). A esto se suma la creciente demanda de los consumidores por productos más saludables y obtenidos en condiciones respetuosas con el medio ambiente, lo que pone de relieve que la búsqueda de fuentes alternativas para la alimentación animal se ha convertido en una prioridad global. Una de estas fuentes alternativas podría ser, sin duda, los insectos.

Las dietas para rumiantes suelen suplementarse con lípidos para aumentar su contenido energético, mejorar el rendimiento animal y/o modular la biohidrogenación ruminal, con el objetivo de incrementar el contenido en carne y leche de ácidos grasos (FA) potencialmente beneficiosos para la salud humana. Entre las grasas más utilizadas en la alimentación de rumiantes se encuentran las derivadas de la palma y el aceite de soja (SBO), ambas con elevado impacto ambiental. El aceite de *Tenebrio molitor* (TMO), un lípido de insectos con un perfil insaturado, podría representar una alternativa sostenible.

Esta tesis de máster se llevó a cabo con el objetivo de investigar los efectos de la suplementación de la dieta con aceite de *T. molitor* sobre la fermentación, degradación y biohidrogenación ruminal en ovejas, utilizando tres sustratos con diferentes proporciones F:C, simulando distintos sistemas de alimentación. Los efectos del TMO se compararon con los de ácidos grasos destilados de palma (PFA) y con los del SBO.

Para ello, se realizó un estudio *in vitro* mediante cultivos discontinuos de microorganismos ruminales y la técnica de producción de gas. Se utilizaron tres ovejas canuladas como donantes del inóculo. Se prepararon tres sustratos con distintas relaciones forraje:concentrado (F:C): 100:0 (denominado 100% F), 50:50 (denominado 50:50 F:C), y 10:90 (denominado 10:90 F:C). Cada uno fue suplementado con una fuente lipídica al 2% materia seca: PFA, SBO o TMO.

Las incubaciones se realizaron durante distintos tiempos según el sustrato: 8 h para el 10:90 F:C, 20 h para el 50:50 F:C y 30 h para el 100% F. Posteriormente, se analizaron parámetros indicativos de la fermentación y degradación ruminal (producción de gas,

concentraciones de amoníaco y ácido láctico, producción de ácidos grasos volátiles, producción estimada de CH_4 y CO_2, desaparición de materia seca y fibra, y digestibilidad verdadera *in vitro* del sustrato) y de la biohidrogenación (composición de FA en la digesta ruminal).

Nuestros resultados mostraron que el TMO apenas tuvo efecto sobre la fermentación y degradación ruminal *in vitro* en los tres sustratos. Las diferencias observadas entre las fuentes lipídicas (PFA, SBO y TMO) fueron generalmente bastante pequeñas.

Los efectos del TMO sobre la biohidrogenación ruminal fueron algo más variables dependiendo de la dieta basal, aunque se identificaron algunos patrones similares en los tres sustratos.

Se detectaron proporciones más altas de ácidos grasos de cadena impar (los cuales se asocian con posibles beneficios para la salud en humanos) en la digesta ruminal de los sustratos suplementados con TMO en comparación con PFA. Los resultados fueron similares entre TMO y SBO.

La mayoría de las diferencias observadas se atribuyeron a la composición de ácidos grasos específica de cada fuente lipídica. Estos efectos parecen ocurrir: i) de manera directa (mediante la transferencia de ácidos grasos del suplemento a la digesta, como el 16:0 con PFA, el *cis*-9 18:1 y el 14:0 con TMO, o el *cis*-9 *cis*-12 18:2 con SBO), o ii) indirecta (mediante la biohidrogenación de FA insaturados, como ciertos isómeros del ácido linoleico conjugado (CLA) o ácidos grasos *trans*-monoinsaturados con SBO y TMO, y 10-oxo-18:0 con TMO).

La suplementación con aceite de *T. molitor* aumentó la concentración de *trans*-11 18:1 respecto a PFA, pero también dio lugar a niveles más altos de *trans*-10 18:1 en los sustratos 100% F y 50:50 F:C. No obstante, cabe destacar que en ningún caso se detectó un cambio en la relación *trans*-10/*trans*-11. Patrones similares a los del *trans*-11 18:1 se observaron para el isómero *cis*-9 *trans*-11 CLA en los mismos sustratos (100% F and 50:50). Estos resultados fueron comparables o ligeramente menos favorables que los obtenidos con SBO, aunque en algunos casos SBO produjo mayores incrementos de *trans*-10 18:1 y *trans*-10 *cis*-12 CLA, ambos potencialmente perjudiciales para rumiantes lecheros.

En conjunto, nuestros resultados apoyan el uso del aceite de *T. molitor* como sustituto de PFA y SBO en ovejas, para reducir tanto la competencia por recursos limitados como el impacto ambiental negativo de estas fuentes lipídicas convencionales. Sin embargo, se requieren más estudios para confirmar estos resultados e investigar el potencial del TMO sobre el rendimiento animal y la calidad de los productos (carne y leche), así como otros aspectos relacionados, por ejemplo, con la aplicabilidad en diferentes contextos productivos.

RÉSUMÉ

Le changement climatique, l'instabilité géopolitique et la croissance démographique ont intensifié la concurrence entre l'alimentation humaine, l'alimentation animale et les biocarburants. À cela s'ajoute la demande croissante des consommateurs pour des produits plus sains, obtenus dans des conditions respectueuses de l'environnement. Il devient alors évident que la recherche de sources alternatives pour l'alimentation du bétail est devenue une priorité mondiale. Parmi ces sources alternatives figurent les insectes.

Les régimes alimentaires des ruminants sont généralement supplémentés en lipides pour augmenter leur teneur énergétique, améliorer les performances animales et/ou moduler la biohydrogénation ruminale afin d'augmenter la teneur en acides gras (FA) potentiellement bénéfiques pour la santé humaine dans la viande et le lait. Les graisses les plus couramment utilisées dans l'alimentation des ruminants proviennent de la palme ou de l'huile de soja (SBO), toutes deux associés à fort impact environnemental. L'huile de *Tenebrio molitor* (TMO), un lipide issu d'insectes au profil insaturé, pourrait constituer une alternative potentiellement plus durable.

Ce mémoire de master a été réalisé dans le but d'étudier les effets de la supplémentation diététique avec l'huile de *T. molitor* sur la fermentation, la dégradation et les processus de biohydrogénation ruminale chez les ovins, en utilisant trois substrats présentant différents rapports fourrage:concentré (F:C), simulant différents systèmes d'alimentation. Les effets ont été comparés à ceux obtenus avec un distillat d'acides gras de palme (PFA) et avec la SBO.

Une étude *in vitro* a été menée en utilisant des cultures discontinues de microorganismes du rumen et la technique de production de gaz. Trois brebis canulées ont été utilisées comme donneuses d'inoculum. Trois substrats avec des rapports F:C différents ont été préparés: 100:0 (appelé 100% F), 50:50 (appelé 50:50 F:C) et 10:90 (appelé 10:90 F:C). Chacun a été supplémenté avec 2% de matière sèche de PFA, SBO ou TMO.

Les incubations ont duré 8 h (10:90 F:C), 20 h (50:50 F:C) ou 30 h (100% F), selon le substrat. Plusieurs paramètres indicateurs de la fermentation et de la dégradation ruminale (production de gaz, concentration d'ammoniac et d'acide lactique, production d'FA volatils,

production estimée de CH_4 et CO_2, disparition de matière sèche et fibre, digestibilité vraie *in vitro* du substrat) ainsi que de la biohydrogénation (composition en FA du contenu ruminal) ont ensuite été analysés.

Nos résultats ont montré que le TMO exerce un effet limité sur la fermentation et la dégradation ruminales *in vitro*, indépendamment du substrat utilisé et les différences observées entre les sources lipidiques (PFA, SBO et TMO) étaient généralement assez faibles.

Les effets du TMO sur la biohydrogénation ruminale ont montré davantage de variation selon le régime de base, bien que certains motifs cohérents aient été identifiés. Des proportions plus élevées d'FA à chaîne impaire, potentiellement bénéfiques pour la santé humaine, ont été observées dans le contenu ruminal des substrats supplémentés en TMO comparativement au PFA. Les résultats étaient similaires entre TMO et SBO.

La majorité des différences ont été attribuées à la composition spécifique en FA de chaque source lipidique, agissant: i) directement (transfert d'FA du supplément au rumen, comme le 16:0 avec le PFA, *cis*-9 18:1 et 14:0 avec le TMO, ou *cis*-9 *cis*-12 18:2 avec le SBO), ou ii) indirectement, via la biohydrogénation des FA insaturés apportés par les suppléments comme des concentrations plus élevées de certains isomères de l'acide linoléique conjugué (CLA) ou d'FA *trans*-monoinsaturés avec SBO et TMO, ou 10-oxo-18:0 avec TMO.

La supplémentation en TMO a augmenté la concentration en *trans*-11 18:1 par rapport au PFA. Elle a également conduit à des concentrations plus élevées en *trans*-10 18:1 dans les substrats 100% F et 50:50 F:C. Néanmoins, il convient de souligner qu'aucun changement dans le rapport *trans*-10/*trans*-11 n'a été observé. Des schémas similaires à ceux du *trans*-11 18:1 ont été observées pour le *cis*-9 *trans*-11 CLA. Ces résultats étaient comparables ou légèrement moins favorables à ceux obtenus avec la SBO. Toutefois, dans certains cas, la SBO a entraîné des augmentations plus marquées de *trans*-10 18:1 et *trans*-10 *cis*-12 CLA, deux FA potentiellement néfastes pour les ruminants laitiers.

Dans l'ensemble, nos résultats soutiennent l'utilisation de l'huile de *T. molitor* comme alternative au PFA et à la SBO chez les ovins, afin de réduire à la fois la compétition pour les

ressources limitées et l'impact environnemental de ces sources conventionnelles. Cependant, des études complémentaires sont nécessaires pour confirmer ces résultats et évaluer le potentiel du TMO sur les performances animals et la qualité des produits (viande et lait), ainsi que d'autres aspects liés, par exemple, à l'extrapolation de ces résultats à différents contextes de production.

الملخص

أدّت التغيرات المناخية، وعدم الاستقرار الجيوسياسي، والنمو السكاني إلى زيادة حدة التنافس بين الغذاء والعلف والوقود. علاوة على ذلك، الطلب المتزايد من المستهلكين على المنتجات الأكثر صحية والتي يتم الحصول عليها في ظروف صديقة للبيئة يجعل من السهل فهم أسباب اعتبار البحث عن مصادر علفية بديلة في تغذية الحيوان أولوية عالمية. لذا قد تكون الحشرات واحدة من هذه المصادر العلفية البديلة.

عادةً ما تُضاف الدهون إلى علائق المجترات بهدف زيادة محتواها من الطاقة وتحسين أداء الحيوانات بما يشمل التأثير على عمليات الهدرجة الحيوية في الكرش وزيادة محتوى اللحوم والألبان من الأحماض الدهنية ذات التأثير الإيجابي على صحة المستهلكين. من بين المصادر الدهنية الأكثر استخدامًا في علائق المجترات تلك المستخلصة من نخيل الزيت وزيت فول الصويا، على الرغم من أن كليهما له تأثير بيئي مرتفع. من ثَمّ، قد يكون زيت *Tenebrio molitor* ، وهو دهن مستخرج من الحشرات ويتميز بتركيب دهني غير مشبع، مصدراً علفيًا بديلاً للدهون في تغذية المجترات.

أُجريت هذه الأطروحة بهدف دراسة تأثيرات إضافة زيت *T. molitor* إلى العليقة على التخمر والتحلل بالكرش، وعمليات الهدرجة الحيوية لدى الأغنام، باستخدام ثلاث ركائز ذات نسب مختلفة من الألياف إلى المركز (F:C) لمحاكاة أنظمة تغذية مختلفة. وتمت مقارنة هذه التأثيرات مع تلك الناتجة عن إضافة إما أحماض دهنية مُقطرة من زيت النخيل أو زيت فول الصويا.

ولتحقيق هذا الهدف، أُجريت دراسة معملية (*in vitro*) باستخدام الكائنات الحية الدقيقة بالكرش المستزرعة على دفعات وتقنية إنتاج الغاز. وتم استخدام ثلاث نعاج مزودة بفتحة كرش (كانولا/فيستيولا) كمصدر لسائل الكرش. وتم تحضير ثلاث ركائز تختلف في نسب الألياف الي المركزات (F:C) علي النحو التالي: 100:0 (تسمى 100%F)، و F:C 50:50 (تسمى F:C 50:50)، و F:C 10:90 (تسمى F:C 10:90) وقد تم معاملة كل منها بإحدى مصادر الدهون التالية بنسبة 2% من المادة الجافة: أحماض دهنية مُقطرة من زيت النخيل، زيت فول الصويا، وزيت حشرة *T. molitor*.

واستمرت عمليات التحضين لأوقات مختلفة حسب الركيزة المستخدمة: 8 ساعات للركيزة F:C 10:90، و20 ساعة للركيزة F:C 50:50، و30 ساعة للركيزة F 100%. تم دراسة بعض المقاييس كمؤشرات للتخمر والتحلل بالكرش (مثل إنتاج الغاز، تركيزات الأمونيا وحمض اللاكتيك، إنتاج الأحماض الدهنية الطيارة، والميثان، وثاني أكسيد الكربون، وتحلل المادة الجافة والألياف غير الذائبة في المحلول المتعادل، وقابلية الهضم الحقيقية المقاسة معمليًا)، وكذلك الهدرجة الحيوية (تركيب الأحماض الدهنية في محتوى البلعة الغذائية بالكرش).

أظهرت النتائج أن زيت حشرة *T. molitor* لم يُحدث تأثيرًا كبيرًا على تخمر وتحلل الكرش معمليًا عبر الركائز الثلاثة التي تمت دراستها. وإذ ظهرت فروقات عند مقارنة تأثير المصادر الثلاثة للدهون (أحماض دهنية مقطرة من زيت النخيل، زيت فول الصويا، وزيت حشرة *T. molitor*)، فقد كانت فروقات طفيفة في هذا الصدد.

كان لتأثيرات *T. molitor* على الهدرجة الحيوية في الكرش بعض التباين حسب العليقة الأساسية. ومع ذلك، تم تحديد أنماط متكررة عبر العلائق الثلاث المدروسة.

تم الكشف عن نسب أعلى من الأحماض الدهنية ذات السلسلة الفردية، والتي قد ترتبط بفوائد صحية محتملة للإنسان، في محتوى الكرش للركائز المضاف إليها *T. molitor* مقارنةً بالاحماض الدهنية المقطرة من زيت النخيل. كانت النتائج متشابهة بين *T. molitor* وزيت فول الصويا.

قد نُسبت معظم التأثيرات المرصودة إلى التركيب النوعي للأحماض الدهنية في كل من الاحماض الدهنية المقطرة من زيت النخيل، زيت فول الصويا، وزيت حشرة *T. Molitor* وبدت هذه التأثيرات تحدث: 1) مباشرة من خلال انتقال الأحماض الدهنية من العليقة إلى محتوى الكرش (مثل 16:0 مع الاحماض الدهنية المقطرة من زيت النخيل، و-*cis* 18:1 9 و 14:0 مع *T. molitor*، و 18:2 12-*cis* 9-*cis* مع زيت فول الصويا، 2) بشكل غير مباشر، عن طريق الهدرجة الحيوية للأحماض الدهنية غير المشبعة التي توفرها المصادر الدهنية المضافة (مثل ارتفاع تركيز بعض المركبات الوسطية، كأنواع محددة من مشابهات حمض اللينوليك المترافق (CLA) أو الأحماض الدهنية الأحادية عدم التشبع من النوع ترانس (*trans*-monounsaturated FA) مع زيت فول الصويا وزيت *T. molitor*، و 10-oxo-18:0 مع زيت *T. molitor*.

أدى إدخال زيت *T. molitor* إلى زيادة تركيز 18:1 11-*trans* مقارنةً بالأحماض الدهنية المقطرة من زيت النخيل، ولكنه أيضًا نتج عنه ارتفاع في مستويات 18:1 10-*trans* في ركيزتي F 100% و F:C 50:50. والجدير بالذكر أنه لم تُلاحظ أي تغيّرات في نسبة 11-*trans*-10/*trans* في أي من الحالات. تم تسجيل أنماط مماثلة لتلك الخاصة بـ 18:1 11-*trans* بالنسبة لـ CLA 11-*trans* 9-*cis* في نفس الركائز (F 100% و F:C 50:50). وكانت هذه النتائج مشابهة أو أقل قليلاً من تلك التي تم الحصول عليها مع زيت فول الصويا. ومع ذلك، في بعض الحالات، تسبّب زيت فول الصويا في زيادات أكبر في 18:1 10-*trans* و CLA 12-*cis* 10-*trans*، وكلاهما قد يكون ضارًا للمجترات المُنتجة للحليب.

إجمالاً، تدعم نتائجنا استخدام زيت حشرة *T. molitor* كبديل لكل من الأحماض الدهنية المقطرة من زيت النخيل وزيت فول الصويا في تغذية الأغنام، بهدف التخفيف ليس فقط من التنافس على الموارد المحدودة، ولكن أيضًا من التأثير البيئي الضار للمصادر التقليدية. ومع ذلك، هناك حاجة لإجراء مزيد من الدراسات لتأكيد نتائجنا، وللبحث في إمكانيات *T. molitor* فيما يخص أداء الحيوانات وجودة المنتجات (اللحوم والألبان)، إضافةً إلى نواحٍ أخرى مثل إمكانية تعميم هذه النتائج على النظم الإنتاجية المختلفة.

I. INTRODUCTION

The current scenario of climate change, geopolitical instability and growing population makes the issue of feed-food-fuel competition more relevant than ever. Add to this the consumer demand for healthier products obtained under the most environmentally friendly conditions possible, and it is easy to understand why the search for alternative sources for livestock feeding has become a global priority (Gasco et al., 2020; Halmemies-Beauchet-Filleau et al., 2018; Makkar, 2018).

One of these alternative sources can certainly be insects. Insect farming uses little water, requires little space, has a high conversion efficiency and allows the use of various agricultural by-products and food waste (Grau et al., 2017; Surendra et al., 2016; van Huis, 2016). As a result, industrial interest in insect production has grown significantly in recent years (Pippinato et al., 2020; Toral et al., in press; Veldkamp et al., 2022).

Although the use of insects as ruminant feed is permitted in many countries (e.g., United States, Canada, Brazil, etc.), it is not yet covered by European legislation, as it is a processed animal protein (Renna et al., 2022b, 2023). However, it is important to note that the use of insect fats in ruminant feeding is not restricted (IPIFF, 2024; Renna et al., 2023) and that many insect companies currently market the different insect fractions separately (i.e., the protein-enriched meal, the purified oil, etc.).

Ruminant diets have traditionally been supplemented with lipids to increase their energy content and enhance animal performance, especially in high-yielding animals (Bauman et al., 2003; Palmquist & Jenkins, 2017; Toral et al., in press). More recently, this dietary lipid supplementation has also been used with the aim of modulating ruminal biohydrogenation and thereby increasing the content in meat and milk of potentially healthy fatty acids for consumers (Shingfield et al., 2013; Toral et al., 2018; Wood et al., 2008). Some of the most commonly used fats are those derived from the oil palm plant and the soybean oil. However, both palm and soybean crops and imports have a high environmental impact (Fearnside, 2001; Fitzherbert et al., 2008; Qaim et al., 2020), so it is urgent to find alternative lipid sources.

Tenebrio molitor (mealworm) is one of the most widely produced species by the insect sector and its rearing is strictly regulated to avoid health risks (Gkinali et al., 2022; IPIFF, 2024; Toral et al., in press). Thus, *T. molitor* oil is one of the insect oils with the greatest market potential. This oil has a very unsaturated lipid profile, with a high content of oleic and linoleic acids (Hong et al., 2020; Noyens et al., 2023; Son et al., 2020), which is very favourable for improving the nutritional quality of milk and meat towards a healthier profile for the consumer.

On this basis, it is hypothesised that palm or soybean lipids used commonly in ruminant diets could be replaced by *T. molitor* oil. This would lead to (i) more sustainable production by avoiding the use of crops that lead to environmental and market conflicts and (ii) added value to animal products (milk and meat) by improving their lipid profile.

As a first step towards incorporating this oil into the ruminant diet, it is necessary, first of all, to analyse its effect on the digestive processes in the rumen, since these will later be the main determinants of diet utilisation, animal performance and product quality.

II. BIBLIOGRAPHIC REVIEW

II. Use of lipids in ruminant diets

Lipids are often supplemented into diets to increase dietary energy density, fat-soluble nutrient absorption, milk synthesis efficiency and body lipid deposition (Bauman et al., 2003; NRC, 2001; Palmquist, 1994). However, it has been reported that total dietary lipids should not exceed 6-7%, as a high lipid content can negatively affect the rumen function (Bauman et al., 2003). On the other hand, the use of lipids can beneficially modify the fatty acid (FA) profile of ruminant-derived products, such as meat and milk (Bauman et al., 2003; Savoini et al., 2019). The FA profile of meat and milk is directly influenced by the extent of lipid metabolism in the rumen (Jenkins et al., 2008; Palmquist et al., 1993). Due to this metabolism, the FA profile of the lipids leaving the rumen (mostly saturated fatty acids; SFA) differs significantly from that of the dietary lipids (mostly unsaturated fatty acids; UFA). During the processes occurring in the rumen, ruminal population plays a key role in lipid metabolism by transforming dietary lipids through two successive processes: hydrolysis and biohydrogenation (BH) (Jenkins et al., 2008). The hydrolysis of ester linkages of dietary lipids by microbial lipases is the first step in rumen lipid metabolism, releasing free FA, which are hydrogenated into SFA by rumen microorganisms (Bauman et al., 2003; De Beni Arrigoni et al., 2016; Frutos et al., 2020). It has been suggested that rumen microorganisms perform the BH process to protect themselves from the toxic effects of UFA (De Beni Arrigoni et al., 2016; Maia et al., 2007). Nonetheless, rumen BH is not a complete process (Frutos et al., 2020; Toral et al., in press) and, therefore, several 18:1 and 18:2 isomers, known as BH intermediates, escape the rumen and can subsequently be deposited into meat and milk (Frutos et al., 2020; Palmquist, 2006b; Toral et al., 2024). Figure 1 shows a schematic representation of the main BH pathways of linoleic and α-linolenic acids, which are the main UFA of ruminant diets (Ferlay et al., 2017; Toral et al., 2024).

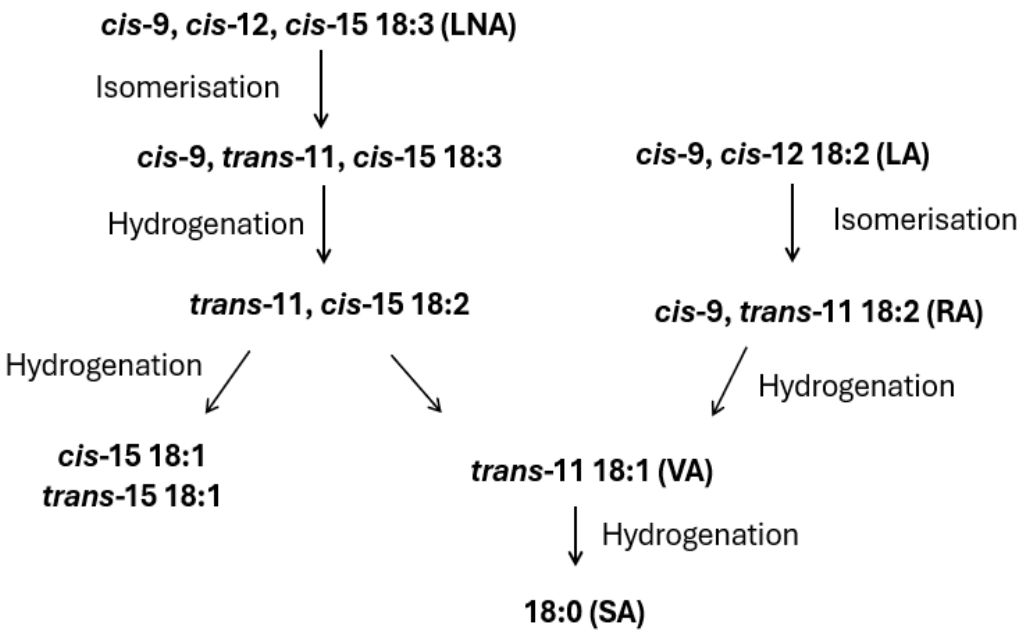

Figure 1. Simplified representation of rumen biohydrogenation pathways of α-linolenic acid (LNA) and linoleic acid (LA), where: RA = rumenic acid; VA = vaccenic acid; SA = stearic acid. Adapted from Bauman et al. (2003) and Jenkins et al. (2008).

Among these intermediate FA, rumen microbial activity produces a pool of conjugated linoleic acid (CLA) isomers, with up to 14 different CLA isomers reported in bovine digesta or *in vitro* contents (Jenkins et al., 2008), including *cis*-9 *trans*-11 18:2 (rumenic acid; RA), which is the most abundant CLA isomer in rumen digesta (Jenkins et al., 2008; Toral et al., 2024). Among *trans* 18:1 intermediates, *trans*-11 18:1 (vaccenic acid; VA) is of particular interest because it is the precursor for the formation of RA (Buccioni et al., 2012; Jenkins et al., 2008). Both VA and RA are BH intermediates of the linoleic acid, while VA comes also from linolenic acid BH (Griinari & Bauman, 1999; Harfoot & Hazlewood, 1997; Toral et al., 2024). The concentrations of RA account for 80-90% of the total CLA isomers in milk and meat fat (Bauman et al., 1999; Ferlay et al., 2017; Frutos et al., 2020). The RA has been reported to possess numerous beneficial effects, such as anti-cancer, antioxidant, and anti-atherogenic properties (Dilzer & Park, 2012; Ip et al., 1995; Lee et al., 1994). Its increase in the rumen is associated with the incomplete BH of linoleic and linolenic acids and can lead to higher flux and deposition of RA in meat and milk (Bauman et al., 1999). In addition, endogenous synthesis, either in the mammary gland or in the muscle, of RA by the

conversion of VA can also affect RA levels in meat and milk (Corl et al., 2001; Frutos et al., 2020; Turpeinen et al., 2002). However, depending on the diet and the rumen environment the main pathways shown in Figure 1 can be altered towards the production of *trans*-10 18:1 and *trans*-10 *cis*-12 CLA, which have been associated with negative productive implications, such as the induction of milk fat depression (Bauman et al., 2003; Toral et al., 2010c, 2016).

Besides being a source of beneficial FA, ruminant-derived products can also contribute, to a greater or lesser extent, to the intake of SFA and *trans* FA in the human diet, some of which can be detrimental to human health (Shingfield et al., 2008, 2013). Alternatively, increasing the UFA content of the human diet at the expense of SFA can promote a healthier human diet (Clifton & Keogh, 2017; Koutsos et al., 2024; Vasilopoulou et al., 2020). To this aim, dietary supplementation of long-chain FA has been shown to reduce the SFA content of ruminant products, as they inhibit the *de novo* synthesis of short- and medium-chain FA in the mammary gland (Bernard et al., 2017; Givens et al., 2009; Palmquist & Griinari, 2006a). However, the extent to which the FA profile of ruminant products can be altered is influenced by the type, inclusion level, and the FA profile of the lipid supplement (Palmquist et al., 1993; Shingfield et al., 2013; Toral et al., 2018).

II.1. CONVENTIONAL SOURCES

Vegetable oils and oilseeds are the most common sources of long-chain FA in ruminant diets and can contribute to decrease SFA content in meat and milk (Glasser et al., 2008; Hervás et al., 2008; Shingfield et al., 2013). Most vegetable oils are derived from beans or seeds, which often yield two useful products: oil and protein-rich meal. Pressing and solvent extraction are two methods used to extract the lipid content of these beans and seeds (Gunstone, 2002; Kaur et al., 2022; Kumar et al., 2017). Palm and palm kernel oils are among the most common SFA-rich lipid sources used in ruminant diets. On the other hand, soybean oil, canola oil, sunflower oil, flaxseed oil, and fish oil are rich sources of polyunsaturated FA (PUFA) that are commonly included in ruminant diets (FEDNA, 2019; Karami et al., 2013; Toral et al., in press).

II.1.1. Palm oil and soybean oil

Palm oil (PO) and soybean oil (SBO) are the most widely produced oils globally. Total production of PO reaches approximately 80 million tonnes and represents more than a third of the world's oil production (FAS-USDA, 2024; Ritchie, 2021). In comparison, SBO accounted for approximately 27% of global vegetable oil production with more than 58 million tonnes (Fang & Kong, 2022; FAO, 2022). Besides, palmitic acid-rich products and SBO are the most used lipid supplements in ruminant diets (Gargouri et al., 2006; Gesteiro et al., 2019; Palmquist & Jenkins, 2017) but the competitive and stable price of PO has made it the most common choice among lipid supplements (Gesteiro et al., 2019).

Concerning the FA content, crude PO has similar proportions of SFA and UFA (approximately 50% SFA and 50% UFA), with palmitic and oleic as main fatty acids (Edem, 2002; Lin, 2002; Sambanthamurthi et al., 2000). In contrast, crude SBO is characterised by its high UFA content (about 84% of the total FA), mainly composed of oleic and linoleic acids (Edem, 2002; Ribeiro et al., 2009; Wang, 2002).

The use of high levels of PO (i.e., 8% dry matter; DM) can have a negative effect on dry matter intake (DMI) and performance in goats (Eknæs et al., 2017). However, these detrimental effects were not reported at 2 and 4% DM in sheep (Flores-Santiago et al., 2022) and at 4% in cows (Suphrap et al., 2019). These discrepancies in DMI among species could be due to differences not only in animal species but also in basal diet, fat source, and inclusion level (Eknæs et al., 2017; Mosley et al., 2007). With respect to SBO, it has been shown that dietary supplementation with SBO at increasing levels (i.e., 3, 6, 9, and 12% DM) linearly decreased DMI in sheep (Lima et al., 2024). Ferreira et al. (2014) reported similar results with 4% DM of SBO in sheep. Conversely, Bouattour et al. (2008) and Gómez-Cortés et al. (2008) found no difference in DMI with SBO inclusion at 2.5% DM in goats and 6% DM in sheep, respectively. The level of incorporation of the oil supplement, dietary energy intake, as well as the fibre content of the basal diet, have been proposed as the main reasons for such differences in DMI (Gómez-Cortés et al., 2008; Lima et al., 2024).

In terms of nutrient digestibility, it has been reported that the addition of PO at 4.1% DM to sheep diet dropped neutral detergent fibre (NDF) digestibility (Manso et al., 2006). The supplementation of SBO at 5% DM to sheep diets showed similar results (Lima et al., 2019). This negative effect could be due to the physical effect of coating the feed particles and the toxic effect on the rumen microorganisms, especially Gram-positive bacteria that degrade cellulose (De Beni Arrigoni et al., 2016; Henderson, 1973). However, Suphrap et al. (2019) obtained that the addition of PO or SBO at 4% DM to the diet of dairy cows had no negative effect on the digestibility of DM, organic matter (OM), or NDF, probably due to the low intake level (i.e., 2% body weight). In beef animals, a significant increase in the digestibility of ether extract (EE) was reported when PO was added at 2.5 and 4.1% DM (Manso et al., 2006) and when SBO was included at 6% DM of sheep diets (van Cleef et al., 2016). This increase in EE digestibility was proposed to be due to the higher digestibility of the added fat and the increased inclusion level (Manso et al., 2006; van Cleef et al., 2016).

Effects on rumen fermentation

In general, the type and the inclusion level of dietary lipids are among the main factors affecting the fermentation process in the rumen (Bauman et al., 2003; Jenkins et al., 2008). Dietary supplementation with SBO was shown to decrease *in vitro* gas production at 8 and 12% DM (Kara et al., 2024). In addition, *in vivo* methane emissions were dropped when PO was added at increasing levels (i.e., 2, 4, and 6% DM; Flores-Santiago et al., 2022), or when SBO was incorporated into sheep diets at 5-6% DM (Gómez-Cortés et al., 2008; Lima et al., 2019). It has been proposed that this reduction in CH_4 production could be due to the rise in the lipid content of the diet at the expense of the NDF content and the subsequent reduction in the retention time of the digesta in the rumen (Flores-Santiago et al., 2022). Similarly, SBO has been shown to decrease ammonia-N concentrations in dairy cows at 2.7% DM (Freitas et al., 2018), which may indicate increased microbial protein synthesis and the boosted delivery of balanced amino acids to the small intestine (Calsamiglia et al., 2010).

Dietary incorporation of PO at 5 and 10% DM was found to have no effect on total volatile fatty acids (VFA) production or the molar proportions of individual VFA *in vitro* (Kim et al., 2014; Matsuba et al., 2019). The supplementation with SBO at 5 and 6% DM showed

similar results regarding total and individual VFA *in vitro* (Gómez-Cortés et al., 2008; Matsuba et al., 2019). Conversely, the addition of SBO at 4% DM into dairy cows' diet was associated with a decrease in the rumen content of total VFA *in vivo* (Yang et al., 2009), which was attributed to the high percentage of UFA that negatively affect rumen fermentation (Buccioni et al., 2012; De Beni Arrigoni et al., 2016; Yang et al., 2009). A significant decrease in the concentrations of acetic, butyric, valeric, caproic, and iso-acids in rumen digesta was also reported when SBO was included at high levels (8 and 12% DM) *in vitro* (Kara et al., 2024). The same authors stated that this reduction was caused by the negative effect of SBO on both carbohydrate and protein ruminal breakdown. In contrast, the molar proportion of propionate was found to be higher when dietary PO was incorporated into sheep diets at 2, 4, and 6% DM (Flores-Santiago et al., 2022), and when SBO was supplemented at 4% DM in dairy cows (Yang et al., 2009). Consequently, the acetate:propionate (A/P) ratio was found to significantly decrease when PO was added at 2, 4, and 6% DM (Flores-Santiago et al., 2022).

Effects on the FA of rumen digesta and meat and milk production

In terms of FA profile of rumen digesta, SBO supplementation can modify the FA profile of rumen digesta by promoting the flux of potentially healthy FA, such as VA, RA, and n-3 FA when added at 3-4% DM (Loor et al., 2002a; Meeprom & Suksombat, 2021; Roy et al., 2017). Consequently, it can increase the milk content of RA, VA, and oleic acid (Bouattour et al., 2008; Girón et al., 2016; Silva et al., 2020). The incorporation of SBO at 1.7 and 3.4% DM into ruminant diets has been associated with a significant rise in milk yield, which could be linked to the higher energy intake (AlZahal et al., 2008). Similarly, dietary PO supplementation at 3 or 8% DM has been associated with an increase in milk fat yield (Eknæs et al., 2017; Fougère et al., 2018), which was explained by the high intake of dietary fat.

II.1.2. Others

Canola and rapeseed oil

Rapeseed, also known as canola (which refers to rapeseed oil containing less than 2% erucic acid), is the third largest cultivated oil crop after PO and SBO (Gunstone, 2004;

Wanasundara et al., 2016). Global production of rapeseed oil and canola oil (CO) exceeded 26 million tonnes in 2022, accounting for approximately 12.6% of total oil production (FAO, 2022). Common rapeseed varieties with higher erucic acid (which is toxic and negatively affects animal performance; EFSA, 2016; Goyal et al., 2021) are cultivated for industrial use in plastics, lubricants, paints, and detergents. On the other hand, CO is produced for food applications (Goyal et al., 2021). According to the Canola Council of Canada (2024), CO contains approximately 61% oleic acid, 19% linoleic acid, 9% α-linolenic acid, and 7% SFA.

Beauchemin & McGinn (2006) reported that CO reduced the DM intake when incorporated at 4.6% DM in the diet of beef cattle. Decreases in DM, NDF and acid detergent fibre (ADF) digestibilities were also found when CO was added *in vivo* to beef cattle diets at 4.6-5% DM (Beauchemin & McGinn, 2006; Zhang et al., 2021). Similarly, the OM digestibility was also decreased when CO was added at 8 and 12% DM *in vitro* (Kara et al., 2024) and at 5% DM in beef cattle diets (Zhang et al., 2021). These negative effects on nutrient intake and digestibility have been attributed to the effect of oil supplementation on rumen microbiota (Zhang et al., 2021).

Dietary CO supplementation at 8 and 12% DM has been shown to decrease gas production *in vitro* (Kara et al., 2024). Lower *in vivo* methane emissions and ammonia-N concentrations were found when CO was added at 4.6-5% DM to beef cattle diets (Beauchemin & McGinn, 2006; Zhang et al., 2021). Similarly, the concentrations of total VFA, iso-valeric and iso-butyric acids, and the A/P ratio were reduced *in vivo* when CO was supplemented into beef cattle diets at 4.6-5% DM (Beauchemin & McGinn, 2006; Zhang et al., 2021), and *in vitro* at 8 and 12% (Kara et al., 2024), while propionate content was increased. The drop in these fermentation parameters was correlated with lowered fibre degradation (Beauchemin & McGinn, 2006). In terms of the FA composition of rumen digesta, dietary CO was reported to increase UFA, CLA, and conjugated linolenic FA when the diet of beef cattle was supplemented at 5% DM *in vivo* (Zhang et al., 2021).

Supplementation of ruminant diets with CO can beneficially affect the FA profile of ruminant-derived products. Milk fat concentrations of oleic acid and CLA were increased by

dietary supplementation with CO at 3.3% DM of dairy cows' diet, whereas proportions of SFA were decreased (DePeters et al., 2001; Loor et al., 2002b).

Sunflower oil

Sunflower oil (SFO) is the fourth most widely produced vegetable oil, with approximately 21.6 million tonnes (Statista, 2024). This oil is characterised by having approximately 15% of SFA, while its high concentration of UFA, mainly composed of oleic acid and linoleic acid, accounts for 85% of the total fat (Akkaya, 2018; Roy et al., 2017).

In terms of intake and rumen fermentation, the addition of SFO showed no effect on feed intake, total VFA, ammonia-N, pH, and *in vitro* DM degradability when included at 2 and 4% DM (Roy et al., 2017; Suphrap et al., 2019; Toral et al., 2010a). In addition, it could enhance the ruminal outflow of VA and RA at 3-4% DM *in vitro* (Roy et al., 2017). It has also been reported that SFO supplementation at 3.7% DM increases the milk fat content of long-chain FA, PUFA, CLA, and n-3 FA in goats (Razzaghi et al., 2015). Hervás et al. (2008) observed a significant increase in milk RA concentrations with dietary supplementation of SFO at 6% DM, without affecting ruminal fermentation, milk production or DMI of dairy sheep. Besides, higher milk content of VA and RA were observed when SFO was added at 2 and 2.5% DM (Toral et al., 2010a, b), and VA and total CLA were linearly increased when SFO was added at increasing levels (i.e., 1.7, 3.4, and 5.1% DM) (Gómez-Cortés et al., 2011b) to dairy sheep diets.

Flaxseed and linseed oil

Flaxseed oil (also known as linseed oil) is extracted from the flax plant (*Linum usitatissimum* L.), an important oilseed crop grown mainly for its oil and fibre contents. The lipid fraction of flaxseed represents about 30-40% of the whole seed, of which about 55% is α-linolenic acid, 19% oleic acid, and 16% linoleic acid, while it contains approximately 9% of SFA (Bernacchia et al., 2014; Ganorkar & Jain, 2013). The inclusion of flaxseed oil in the diet of ruminants has been reported to increase the proportion of propionate while decreasing the proportion of acetate and the A/P ratio when added at 4% DM (Kholif et al., 2018; Pi et

al., 2019). It can also promote the total UFA content in the ruminal fluid, particularly VA, RA, and α-linolenic acid, at 4 and 6% DM *in vivo* (Bessa et al., 2007; Pi et al., 2019).

Dietary flaxseed oil supplementation at 2-4% DM has been shown to reduce the level of SFA in milk fat and increase milk production and the concentration of some beneficial FA, such as α-linolenic acid, VA, and CLA, without adversely affecting performance and milk composition (Kholif et al., 2018; Pi et al., 2016; Suksombat et al., 2016).

Fish oil

Fish oil (FO), also used as animal fat in ruminant diets, can be obtained from fish byproducts, as well as from the processing and pressing of whole fish (FEDNA, 2019). The FO is rich in n-3 long-chain FA with more than 20 carbon atoms, particularly C20:5n-3 and C22:6n-3, and poorer in n-6 FA (AbuGhazaleh et al., 2002; FEDNA, 2019; Shingfield et al., 2008).

Dietary FO supplementation can reduce DMI and nutrient digestibility when added to the diet of dairy cows at 2-3% DM (AbuGhazaleh et al., 2002; Donovan et al., 2000). This reduction in DMI was attributed to a decrease in ruminal degradation of the fibre content, because of the high UFA concentration in FO and the palatability of the oil. The addition of FO at lower levels (i.e., 1-2% DM) has been reported to have no effect on VFA production *in vivo* (Hernández-García et al., 2017; Toral et al., 2010a). Concerning rumen digesta FA, several studies have demonstrated incomplete hydrogenation of UFA when dietary FO was included in ruminant diets at 1-2% DM (AbuGhazaleh et al., 2002; Vargas et al., 2017). Consequently, the flow of BH intermediate FA to the abomasum and therefore to milk and meat can be increased (Shingfield et al., 2008). Overall, the incorporation of FO at 2-3% DM into dairy cows' diet has been associated with an important increase in the milk fat concentrations of UFA, especially VA, RA, and n-3 FA (AbuGhazaleh et al., 2002; Allred et al., 2006; Donovan et al., 2000). Likewise, Ferreira et al. (2014) reported that FO supplementation with increasing levels up to 7.5 g/kg DM enriched the meat content of UFA. However, it should be noted that the inclusion of FO at 2% DM in ruminant diets was associated with milk fat depression (Frutos et al., 2018; Toral et al., 2016).

II.2. NON-CONVENTIONAL SOURCES

In the last decades, the competition among food, feed, and fuel production, and the higher need for land, water, labour, and capital has expanded dramatically. This competition has been driven by: 1) the ever-increasing demand for bioenergy and the amount of human-edible food used for livestock and viceversa (Makkar, 2016; Muscat et al., 2020), and 2) the shift in ruminant feeding systems towards the use of intensive feeding systems, which has raised the reliance on human food crops to meet the energy and protein requirements of ruminants (Karlsson & Röös, 2019; Van Zanten et al., 2019). As a result, palm and soybean cultivation has increased to meet the animal needs of energy and protein, which favours tropical deforestation, as they are mainly cultivated at the expense of forest and pastureland (Fang & Kong, 2022; Ritchie, 2021).

To overcome this competition for scarce resources, using non-conventional ingredients in animal diets might reduce dependence on human food crops. Consequently, nowadays, there is an increasing interest in using for example insects and their products (meals and oils) as alternatives to traditional plant-based feeds in animal diets (Renna et al., 2022b; Siddiqui et al., 2024; Toral et al., in press).

II.2.1. Insects as a novel feed

Insects are members of the phylum Arthropoda, which includes more than 1.5 million animal species worldwide, of which more than two-thirds are insects (van Huis et al., 2013; Van Raamsdonk et al., 2017). Insects have been proposed as an alternative protein source that can contribute to ensuring global food security (van Huis et al., 2013; Veldkamp et al., 2012). Because of their capacity to develop on comparatively poor diets and produce high-quality protein, insects have attracted much interest as food and feed ingredients (Hawkey et al., 2021; Toral et al., in press). In addition, they are also a good source of lipids (Aguilar, 2021; Cito et al., 2017; Prachumchai & Cherdthong, 2023). However, it should be noted that the chemical composition of insects depends on a number of factors (species, sex, diet, etc.) which leads to a high variability (Cruz et al., 2025; Dossey et al., 2016).

II.2.1.1. Insect farming

Insect farming is gaining potential due to the efficiency and sustainability of its production (Gasco et al., 2020; van Huis et al., 2013). Globally, about 1.3 billion tonnes of edible food are wasted and lost annually throughout the entire supply chain (Fox & Fimeche, 2013; Veldkamp et al., 2012). Insects are able to consume these residues and other low-value organic wastes to produce high-value proteins with a high feed conversion efficiency (Grau et al., 2017; Ramos-Elorduy et al., 2002; van Huis, 2016).

The short life cycle of insects (Ramos-Elorduy et al., 2002; van Huis et al., 2013), rapid reproduction of large populations (Makkar et al., 2014; van Huis et al., 2013), and low territorial requirements (Madau et al., 2020; Salomone et al., 2017) have attracted attention to insect mass production. As a result, in 2020, the number of companies dedicated to the production, processing, and sale of insects and their derived products increased extraordinarily in the European Union (EU), with *Tenebrio molitor* and *Acheta domesticus* being the most sold insects (Pippinato et al., 2020).

II.2.1.2. Insect meals

Insect meals are the product obtained after processing (i.e., cleaning, drying, heating, and grinding) of whole insects (Veldkamp et al., 2012). Insect meals are considered promising and sustainable alternatives to plant proteins used in ruminant diets, such as soybean meal (AbdelHakeam et al., 2024; Renna et al., 2023; Toral et al., 2022), and their use in ruminant farming could contribute to limit the impact of ruminant farming (Gasco et al., 2020). In fact, the crude protein (CP) content of commonly used insects for food and feed is comparable to that of soybean (Ahmed et al., 2021; Hawkey et al., 2021). However, the use of insect meals in ruminant diets is still restricted in some high-come countries because they are considered processed-animal-proteins, that are related to the potential risk of bovine spongiform encephalopathy (also known as mad cow disease) (Directive No. 1069/2009 and its implementing Regulation No. 142/2011) (Renna et al., 2022b, 2023).

Regarding the effects of insect meals on diet utilisation, some studies have shown that the partial replacement of 25-30% of soybean meal with insect meals from *Acheta*

domesticus, *Brachytrupes portentosus*, *Gryllus bimaculatus*, and *Bombyx mori* has no adverse effect on *in vitro* fermentation or nutrient degradability (Ahmed et al., 2021; Rashmi et al., 2022). Moreover, an *in vivo* study conducted by AbdelHakeam et al. (2024) suggested that replacing 10, 20, and 30% of soybean meal with oriental hornet meal linearly enhanced the digestibility of DM, OM, CP, and EE probably due to the low lipid content of this meal. However, higher inclusion levels of full-fat insect meal (i.e., 50 or 100% replacement of soybean meal) led to decreased *in vitro* nutrient degradability in Jayanegara et al. (2017a). This reduction of fermentation and degradability can most probably be related to the high EE content and the low degradability of the chitin fraction (a polysaccharide obtained by deproteinising insect powder) of insect meals (Jayanegara et al., 2017a; Renna et al., 2022a). Therefore, defatting processes have been suggested as a good strategy to reduce the negative impact on rumen fermentation and degradability of nutrients (Phesatcha et al., 2022; Rashmi et al., 2018), besides of reducing the risk of oxidation and rancidity (Renna et al., 2023; Veldkamp et al., 2012). Regarding the ruminal degradation of the protein, the larval meal of *Hermetia illucens*, *Tenebrio molitor*, and *Notonecta spp*. showed similar values than those of soybean meal and higher than those observed in fish meal (Robles-Jimenez et al., 2022), which could promote the ruminal microbial growth (Pathak, 2008; Renna et al., 2023). In this line, AbdelHakeam et al. (2024) stated that the partial replacement of 10, 20, and 30% of soybean meal with oriental hornet meal promoted the ruminal content of microbial protein and total protozoa count in insect-fed lambs compared with the control.

Other studies have demonstrated the ability of insect meals from *Acheta domesticus*, *Alphitobius diaperinus*, *Blatta lateralis*, *Grylloides sigillatus*, *Gryllus bimaculatus*, *Hermetia illucens*, *Musca domestica*, and *Tenebrio molitor* to reduce *in vitro* gas production when totally replaced soybean meal or incubated as the sole substrate compared to soybean meal (Jayanegara et al., 2017a; Renna et al., 2022a). A significant drop in the total VFA production was also observed when *Acheta domesticus* meal completely replaced soybean meal in the diet *in vivo* (i.e., 10% DM). These results were probably due to the high lipid fraction of the insect used and the relatively high UFA content (Hervás et al., 2024). Similarly, Jayanegara et al. (2017a) indicated that higher inclusion levels of full-fat of *Hermetia illucens* meal (i.e.,

50 or 100% replacement of soybean meal) significantly decreased *in vitro* VFA production. Decreases in VFA production were also reported by Renna et al. (2022a) when they incubated eight full-fat insect meals (i.e., *Acheta domesticus*, *Alphitobius diaperinus*, *Blatta lateralis*, *Grylloides sigillatus*, *Gryllus bimaculatus*, *Hermetia illucens*, *Musca domestica*, and *Tenebrio molitor*) with ovine rumen inoculum and as the sole substrate compared to soybean, rapeseed, and sunflower meal.

When moving to the effect of insect meals on rumen BH and, consequently, on the FA profile of ruminant products, the available studies are scarce (Toral et al., in press). Hervás et al. (2024) indicated that the complete replacement of 10% DM soybean meal with *Acheta domesticus* meal modulated the rumen BH process and significantly promoted the rumen digesta content of some bioactive FA, such as VA and RA, but also increased the level of *trans*-10 18:1. Renna et al. (2022a) reported that a full-fat meal of *Acheta domesticus*, *Alphitobius diaperinus*, *Grylloides sigillatus*, *Gryllus bimaculatus*, and *Tenebrio molitor* used as sole *in vitro* substrate resulted in greater ruminal levels of potentially healthy FA, such as VA, RA, and linoleic acid, compared to soybean, rapeseed, and sunflower meals. These positive effects were probably due not only to the higher total FA content (more than 10 times greater) of full-fat insect meals compared to plant-based meals (Renna et al., 2022a) but also to the lower BH of PUFA from insect meals, as some of them (i.e., *Alphitobius diaperinus*, *Grylloides sigillatus*, and *Tenebrio molitor*) were rich in PUFA, that prevents from extensive BH (Buccioni et al., 2012; De Beni Arrigoni et al., 2016).

II.2.1.3. Insect lipids

It is probably worth mentioning, first of all, that insect oils have no restrictions to be used in ruminant diets (IPIFF, 2024; Renna et al., 2023).

Insect lipids, which can be obtained by defatting edible insects, are an important insect-derived product that shows promise as a replacement for conventional vegetable oils used in ruminant diets, such as PO and SBO (Hervás et al., 2022; Rastello et al., 2024; Toral et al., in press). The lipid fraction is the second-largest component of edible insects after protein (Benzertiha et al., 2020; Rumpold & Schlüter, 2013) and both the lipid content and

the FA profile of insects vary significantly depending on insect species, developmental stage, season, and their basal diet (Nowak et al., 2016; Sánchez-Muros et al., 2014). According to Dossey et al. (2016), the larval stages of holometabolous insects, such as mealworms, butterflies, moths, and flies, tend to have greater lipid content than their respective adult stages or when compared with the majority of hemimetabolous insects, such as crickets and grasshoppers. The primary forms of insect lipids are triglycerides (energy reserve), phospholipids, steroids, and waxes (Aguilar, 2021; Benzertiha et al., 2020) and are found in liquid phase at room temperature in most insect species (e.g., house fly maggots, mealworms, and house crickets) due to their high UFA content (i.e., more than 60% of total FA; Makkar et al., 2014). For this reason, they are known as insect oils. Nevertheless, the lipid fraction of black soldier fly larvae contains about 57-75% SFA (Makkar et al., 2014; Sosa & Fogliano, 2017; Toral et al., in press) and is solid at room temperature.

In ruminants, although few studies have investigated the use of insect-derived lipids as energy supplement (Palupi et al., 2025; Toral et al., in press), lipids from mealworms, crickets and black soldier fly have shown promising *in vitro* effects in decreasing methane emissions when added at 4-5% DM (Jayanegara et al., 2020; Prachumchai & Cherdthong, 2023). This lower methane has been linked to the reduction in the number or activity of methanogens, along with a partial removal of protozoa, and a drop in the degradation and fermentation involved in the release of hydrogen (Jayanegara et al., 2020; Prachumchai & Cherdthong, 2023; Thirumalaisamy et al., 2020).

Regarding their effects on nutrient degradability, insect oils have been associated with negative effects on *in vitro* rumen degradation of nutrients when included in the diet at high levels (e.g., 3% DM or more; Jayanegara et al., 2021; Thirumalaisamy et al., 2020). In the study conducted by Jayanegara et al. (2021), increasing inclusion levels of maggot oil (i.e., 3, 4, and 5% DM) linearly decreased *in vitro* DM and OM degradation. The same trend in DM degradation was observed when silkworm pupae oil was added at 4 and 5% DM to different *in vitro* diets (with high-fibre, medium-concentrate, total mixed ration; TMR, and high-concentrate substrates) (Thirumalaisamy et al., 2020). This decrease in degradation is

related to the lipid supplementation, especially at higher levels, which negatively affects rumen fermentation and carbohydrate digestion (Jayanegara et al., 2021; Patra, 2013).

Concerning the direct effect of insect lipids on rumen BH, the available literature is actually limited. Hervás et al. (2022), studying diet supplementation with house cricket, black soldier fly and silkworm oils, observed that the first one (house cricket oil) at 2% DM significantly increased the accumulation of VA in the rumen digesta. In addition, they also stated that insect oils had no effect on the levels of *trans*-10 18:1 (an undesirable FA; Bauchart et al., 2007; Frutos et al., 2020; Shingfield et al., 2008), while SBO tended to affect *trans*-10 18:1 concentration, suggesting a shift in the ruminal BH pathways. These findings reinforce the possibility of replacing conventional lipids such as PO and SBO with insect-derived lipids.

Although there are few studies investigating the potential use of insect oils to modulate the FA profile of ruminant derived products, they have been shown to be able to modify the FA profile of milk fat (Nekrasov et al., 2022; Rastello et al., 2024). In the study conducted by Nekrasov et al. (2022), dietary supplementation with *Hermetia illucens* lipid at 10 g/cow/d increased the milk fat content of total monounsaturated FA (MUFA) and PUFA compared to unsupplemented cows, but the insect lipid also increased the total SFA fraction. Similarly, Rastello et al. (2024) found that the inclusion of *H. illucens* fat at 3% (as fed basis) to cows' diet significantly enhanced the levels of total *trans* 18:1 and *trans* 18:2 isomers in milk fat, while decreasing the 16:0, 18:0, and total MUFA content compared with cows fed a comparable level of palm oil. These effects were proposed to be, to some extent, the result of differences in the FA profile of the used lipid supplements (Rastello et al., 2024).

II.2.1.4. *Tenebrio molitor*

Tenebrio molitor is a member of the family Tenebrionidae and its larval stage is commonly known as mealworm. It has a short life cycle and high reproductive rates (Errico et al., 2022; Langston et al., 2024; Thévenot et al., 2018) and its development consists of four life stages: egg, larva, pupa, and adult stage. Females can lay up to 500 eggs and the hatching process begins within 10-12 days after the egg laying. Then, the larvae usually take

3-4 months to reach the adult stage at room temperature (Makkar et al., 2014). Afterwards, the pupae develop within 20 days and adults usually live for 2-3 months (Hill, 2002).

II.2.1.4.1. Farming

Recently, the large-scale production of mealworms has received increased attention, not only because they are the most widely bred, reared, sold, and consumed edible insect larvae in Asian and European countries, but also because they are the first edible insect to be approved by the European Food Safety Authority as an innovative food (Anusha & Negi, 2023; Errico et al., 2022). Their production accounts for one-third of all insect production companies in the EU (Pippinato et al., 2020).

Mealworms have a high production capacity on the industrial scale (Jin et at., 2016; Mancini et al., 2020) because their edible content is approximately 100% (Errico et al., 2022) and, in addition, they can be sold in live form, canned, dried, or even in powdered form (Li et al., 2013; Siddiqui et al., 2024; Tran et al., 2019). All this, together with their valuable protein profile (about 52% in peeled and hot-air dried mealworm larvae and 71% in defatted larvae; Son et al., 2020), have led to their industrial production as a feed for several animal species.

II.2.1.4.2. Nutritional value and FA profile

The nutritional value of *T. molitor* varies considerably depending on, among other factors, the type of feeding substrate, larval size, rearing conditions, and the life cycle stage (Ramos-Elorduy et al., 2002; Toviho & Bársony, 2022). Larger larvae have been shown to have proportionally higher DM, CP, ash, and crude fibre contents than normal or small-sized larvae. In contrast, small larvae have higher percentages of chitin, nitrogen-free extract, and EE than large larvae (Toviho & Bársony, 2022). The mean DM content of mealworms ranges from 28.3 to 35.3% (Noyens et al., 2023). Makkar et al. (2014) and Syahrulawal et al. (2023) reported that the average CP fraction in *T. molitor* is about 52%, although it varies mainly with the feeding substrate (Harsányi et al., 2020; Zhang et al., 2019). Lipids are a major component in *T. molitor* larvae (Aguilar, 2021; Cito et al., 2017), representing approximately 35% DM. Macro- and microminerals constitute around 4% DM (Barker et al., 1998; Liu et al.,

2020; Zhao et al., 2016) and the chitin content ranges from 6.1 to 7.8% DM and non-fibrous carbohydrates from 14 to 19.4% DM (Laroche et al., 2019; Noyens et al., 2023).

Concerning the FA profile of mealworm oil, the major FA are oleic, linoleic, and palmitic (Lee et al., 2022; Ugur et al., 2021; Verheyen et al., 2023). The UFA accounts for more than 75% of the total FA, of which PUFA represents 29% and MUFA 46% (Cito et al., 2017; van Huis et al., 2013). Among PUFA, mealworms have high content of n-6 PUFA (Cito et al., 2017; van Huis et al., 2013). Table 1 shows the fatty acid composition of mealworm oil.

Table 1. Fatty acid composition[1] of *T. molitor* larvae oil (g/100 g FA). Adapted from Noyens et al. (2023), Son et al. (2020), and Wu et al. (2020).

Item	g/100 g FA
8:0	0.01 - 0.19
10:0	0.01 - 0.14
12:0	0.15 - 0.32
13:0	0.05 - 0.1
14:0	1.65 - 4.86
cis-9 14:1	0.01
16:0	10.96 - 22.7
cis-9 16:1	0.48 - 3.01
17:0	0.06 - 0.26
18:0	0.68 - 2.87
cis-9 18:1	27.1 - 45.8
18:2n-6	26.3 - 51.5
18:3n-3	0.4 - 2.98
20:0	0.05 - 0.25
20:1	0.04 - 0.1
20:2n-6	0.1
SFA	17.4 - 28.2
MUFA	27.5 - 46.2
PUFA	19.8 - 51.5
UFA	66 - 80.2

[1] SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, UFA = unsaturated fatty acids.

II.2.1.4.3. Effects on rumen fermentation and animal performance

Few studies have investigated the effect of mealworm meal on the rumen fermentation process. Renna et al. (2022a) indicated that *in vitro* total gas production was decreased when full-fat *Tenebrio molitor* (TM) meal was incubated for 24 h as sole substrate compared with similar amounts of soybean, rapeseed, and sunflower meals. A reduction in the *in vitro* total gas production was also observed when it was incubated under similar conditions (i.e., as the sole substrate) but for 96 h against both soybean meal and fish meal (Robles-Jimenez et al., 2022). In addition, full-fat TM meal significantly reduced *in vitro* CH_4 emissions when compared to some plant-based meals, but also total VFA production and acetate and propionate proportions (Renna et al., 2022a), which could be due to the high content of CP and EE (Renna et al., 2022a; Robles-Jimenez et al., 2022). Moreover, the chitin content in insect meals could also contribute to the lower gas and VFA production (Del Valle et al., 2017; Jayanegara et al., 2017b).

Concerning *in vitro* disappearance of nutrients, *in vitro* incubation of full-fat TM meal as the sole substrate resulted in a significant decrease in the degradation of DM and OM compared to soybean meal (Renna et al., 2022a; Robles-Jimenez et al., 2022). In addition, Toral et al. (2022) observed that TM meal had higher non-degraded protein intestinal digestibility (i.e., 78 for TM vs. 68% for soybean meal) and lower ruminal N degradation compared to soybean meal when TM meal was incubated for 16 h both *in vitro* using 500 mg DM and *in situ* with 6 g DM. Enhancing the proportion of protein that escapes ruminal degradation, assuming high intestinal digestibility, has the potential to improve ruminant performance (Haryanto, 2014; Renna et al., 2022a; Rotta et al., 2023).

On the contrary, Hanönü et al. (2024) reported that the addition of TM meal at 0.5, 1, and 1.5% DM to alfalfa hay resulted in a linear increase of *in vitro* gas production and OM degradation compared to a diet containing only alfalfa hay, while no changes in CH_4 emissions were observed. This rise in gas production and DM degradation was mainly due to the higher digestibility of TM meal compared with the alfalfa hay. Robles-Jimenez et al. (2025) also reported positive effects regarding *in vitro* DM disappearance when full-fat TM meal was included at 60 g/kg DM in a TMR of fattening lambs. They also stated that full-fat

TM meal had higher total tract apparent digestibility of DM and OM when compared with soybean meal.

Regarding the effect of *T. molitor* oil on *in vitro* rumen fermentation kinetics, no differences in gas production, ammonia-N concentrations, and total VFA production were observed when it was added at 5% DM to forage- and concentrate-based diets (Jayanegara et al., 2020). However, methane, and DM and OM degradation were significantly decreased, which was attributed to the inhibitory effect of the insect lipid on the activity of methanogens and on nutrient degradation and fermentation (Beauchemin et al., 2009; Patra et al., 2017).

In terms of the FA profile of rumen digesta, the article by Renna et al. (2022a) is, to our knowledge, the only study available nowadays. They reported that, when incubated for 24 h, full-fat TM meal produced higher levels of total MUFA, PUFA, and CLA than soybean, rapeseed, and sunflower meals, while reduced the SFA content. This enhancement in MUFA and PUFA was attributed to their very high content in the lipid fraction of full-fat TM meal compared to that in the plant-based meals (Renna et al., 2022a).

Robles-Jimenez et al. (2025) supplemented the diet of growing lambs with 60 g/kg DM of full-fat TM meal and obtained similar DMI when compared to lambs fed diets supplemented with fishmeal, but lower than that of lambs fed soybean meal. In addition, average daily gain was decreased in lambs fed the TM relative to lambs fed fishmeal and soybean meal, which was attributed to the high chitin and lipid content of the TM used.

II.2.1.4.4. Effects on the fatty acid composition of ruminant-derived products

To the best of our knowledge, there are no published articles discussing the potential effect of *T. molitor* on the FA profile of ruminant-derived products. However, the FA composition of the rumen digesta reported by Renna et al. (2022a) when 600 mg DM of full-fat TM meal was incubated as the sole substrate, with high PUFA contents, would support the potential of this feed to improve the quality of ruminants' meat and milk. Nevertheless, further *in vitro* and *in vivo* studies are needed to confirm these findings.

Finally, it is noteworthy the recommendation by Toral et al. (in press) about the use of insect fats in ruminant feeding: when the aim is to modulate the FA composition of animal-derived products, insect-derived lipids should replace fats with comparable or lesser levels of unsaturation. For example, *T. molitor* oil could replace UFA-rich sources such as soybean, rapeseed, cottonseed, and sunflower oils, while *H. illucens* should substitute SFA-rich fats, such as coconut and palm kernel oils.

III. AIMS

As already mentioned in the Introduction, before incorporating any feed into the ruminant diet, it is necessary, as a first step, to analyse its effect on the digestive processes in the rumen, because these will later be the main determinants of diet utilisation and subsequent animal performance, and, in the case of lipid composition, product quality.

Therefore, this MSc thesis was carried out with the aim of investigating the effect of dietary supplementation with *T. molitor* oil on ruminal fermentation, degradation and biohydrogenation processes in sheep. This effect was compared to that of diet supplementation with other conventional fats, namely, soybean oil or palm distillate fatty acids.

Moreover, given that ruminal processes are affected by the forage:concentrate ratio of the diet (McAllister & Newbold, 2008; Russell & Rychlik, 2001; Van Soest, 1994), we decided investigating the incorporation of *T. molitor* oil to replace either soybean oil or palm distillate fatty acids, into three diets with different F:C ratios.

Thus, to meet the aims of this study, we conducted an *in vitro* assay in which we chose a dose of lipid supplementation normally used under practical conditions, 2% DM, and three diets with 100:0, 50:50 or 10:90 F:C ratios, to simulate different ruminant feeding systems. Then, we analysed some parameters as indicative of rumen fermentation and degradation (e.g., gas production, ammonia and volatile FA concentration, DM disappearance; DMD, NDF disappearance; NDFD) and biohydrogenation (rumen digesta FA composition).

To the best of our knowledge, and as can be confirmed in the Literature review, the information available on this subject is extremely scarce.

IV. MATERIAL AND METHODS

IV.1. ANIMALS AND DIETS

All procedures with experimental animals were conducted at the Ruminant Research Unit from the *Instituto de Ganadería de Montaña* (IGM, CSIC-University of León), following the Spanish and European Union regulations [Royal Decree 53/2013 (BOE, 2013) and Council Directive 2010/63/EU (EU, 2010)] for the protection of animals used for experimental purposes.

Three mature Assaf ewes (average body weight = 77.5 ± 6.02 kg), neither pregnant nor lactating and equipped with a ruminal cannula (40 mm internal diameter, patented Utility Model No. ES1259271), were used as ruminal fluid donors. Ewes were housed in individual cages three weeks prior to the ruminal fluid collection, and were fed once daily, at 08:30 h, with oat hay offered *ad libitum* and a concentrate to meet 1.1 their estimated maintenance requirements for energy (INRA, 2018). The concentrate was composed of (% of fresh matter [FM]) 40% alfalfa pellets, 14.5% barley grain, 14% corn grain, 7.8% cereal straw, 7% wheat grain, 6.5% wheat bran, 5% soybean meal (470 g CP/kg), 2% sugar beet molasses, 1.2% $CaCO_3$, 1% animal fat, 0.5% $NaHCO_3$, 0.3% salt, and 0.2% vitamin-mineral corrector. Table 2 shows the chemical composition of the offered concentrate and hay. Ewes always had free access to fresh water and mineral blocks (Tegablock, INATEGA S.L., Corbillos de la Sobarriba, León, Spain).

Table 2. Chemical composition of the concentrate and hay offered to the ewes.

Item	Concentrate feed	Oat hay
Chemical composition, % DM[1]		
DM	95.58	94.17
CP	13.02	7.79
EE	3.27	2.22
NDF	29.11	54.58
ADF	16.36	31.07
ADL	2.80	3.14
Ash	10.05	7.30

[1] DM = dry matter, CP = crude protein, EE = ether extract, NDF = neutral detergent fibre, ADF = acid detergent fibre, and ADL = acid detergent lignin.

Substrates for *in vitro* incubations

Three substrates with different F:C ratios (100:0, 50:50, and 10:90) were formulated to simulate different feeding systems: from a diet based entirely on forage (100% F), to a diet commonly used for dairy ewes (50:50 F:C), or a diet for fattening lambs (10:90 F:C).

To prepare the 100% F substrate, a medium-quality grass hay was utilised. The 50:50 F:C substrate consisted of a TMR based on (% of FM): 50% dehydrated alfalfa (particle size > 4 cm), 14% corn grain, 19% barley grain, 15% soybean meal solvent (440 g CP/kg), 5% sugar beet pulp, 4% sugar beet molasses, 2% vitamin-mineral supplement (MACROFAC Rumiantes, UP911755139; DSM Nutritional Products S.A., Madrid, Spain). The 10:90 F:C substrate was prepared by mixing 10% wheat straw with 90% of a concentrate containing (% FM): 23% corn grain, 17% barley grain, 16.5% soybean (470 g CP/kg), 15% sunflower cake, 10% wheat, 7% corn gluten feed, 6% wheat bran, 2% sugar beet molasses, 1.8% $CaCO_3$, 0.5% animal fat, 0.5% $NaHCO_3$, 0.5% salt, and 0.2% vitamin-mineral supplement.

IV.2. EXPERIMENTAL TREATMENTS

Each of the 3 incubation substrates (100% F, 50:50 F:C and 10:90 F:C) was supplemented with 3 different lipid sources at 2% DM:

- Palm distillate fatty acids, which were obtained from SOLAFAM 440 (AFAMSA S.A., Mos, Pontevedra, Spain).
- Soybean oil, which was obtained from OLI-BEEF (INATEGA S.L., Corbillos de la Sobarriba, León, Spain).
- *T. molitor* oil, which was obtained from Tebrio Group S.L. (Doñinos de Salamanca, Salamanca, Spain).

This resulted in the following experimental treatments:

- 100% F substrate + Palm distillate fatty acids.
- 100% F substrate + Soybean oil.
- 100% F substrate + *T. molitor* oil.

- 50:50 F:C substrate + Palm distillate fatty acids.
- 50:50 F:C substrate + Soybean oil.
- 50:50 F:C substrate + *T. molitor* oil.

- 10:90 F:C substrate + Palm distillate fatty acids.
- 10:90 F:C substrate + Soybean oil.
- 10:90 F:C substrate + *T. molitor* oil.

Although it is tempting to think of a 3 x 3 factorial design, this is not possible in this case. Each substrate, due to its own characteristics, was incubated for a different time to better approximate the retention time of the feed in the rumen. Thus, different incubation times prevent statistical analysis following a factorial model, as the main factor of variation would actually be the incubation time.

IV.3. BATCH CULTURES OF RUMEN MICROORGANISMS

Batch cultures of rumen microorganisms and the gas production technique were used following Hervás et al. (2005). The culture medium was prepared using macro- and micromineral solutions, a bicarbonate buffer solution, and resazurin (Goering & Van Soest, 1970). To ensure its reduction, the medium was kept in a water bath at 39.5 °C with a constant flow of CO_2 for about 45 minutes. The chemical composition of the culture medium is shown in Table 3.

Table 3. Chemical composition of the culture medium.

Solutions (Chemical compounds)		Partial concentration (/L)	Final solution (/L)
Macromineral solution	(mL)		208.1
$Na_2HPO_4 \cdot 12H_2O$	(g)	9.45	
KH_2PO_4	(g)	6.20	
$MgSO_4 \cdot 7H_2O$	(g)	0.60	
Micromineral solution	(mL)		0.104
$CaCl_2$ $2H_2O$	(g)	3.30	
$MnCl_2$ $4H_2O$	(g)	2.50	
$CoCl_2$ $6H_2O$	(g)	0.30	
$FeCl_3$ $6H_2O$	(g)	2.00	
NH_4CO_3	(g)	4.0	
$NaHCO_3$	(g)	35	
Reduction solution	(mL)		62.4
Cysteine HCl	(g)	6.25	
NaOH 1M	(mL)	40	
Na_2S $9H_2O$	(g)	6.25	
Resazurin solution	(mL)		1.040
Resazurin	(g)	0.01	

On each collection day, animals were fed at 08:30 h as usual. Subsequently, orts were discarded (if any) at 09:30 h, water was blocked at 10:30 h, and the inoculum was collected at 11:30 h. The ruminal inoculum was obtained through the cannula and was immediately filtered by two layers of gauze. Then, it was quickly transported to the laboratory in pre-heated vacuum flasks to try to keep anaerobic conditions and temperature. Once in the laboratory, it was filtered again using a nylon membrane (250 µm; Fisher Scientific S.L., Madrid, Spain).

Then, the inoculum and the culture medium were mixed in a 1:4 ratio and 50 mL of the final mixture were dosed into each 125 mL incubation bottle. All bottles contained 500 mg of the corresponding substrate (which had been weighed previously) and oil.

Each of the three oil supplements was dissolved in ethanol 98% (i.e., 2.5 g in 25 mL ethanol). The solutions were sonicated at 24 kHz and 150 W for 3 cycles of 30 seconds to ensure homogenisation (Ultraschallprozessor UP200H, Stahnsdorf, Germany). Then, 20 g/kg DM of each dissolved oil was dosed into the incubation bottles just before starting the incubation, whereas only ethanol was added to the blanks.

Once the substrates, the lipid supplements and the inoculum + culture medium were prepared, the bottles were tightly sealed with rubber stoppers and aluminium rings, shaken, and incubated (UFP 500; Memmert, Schwabach, Germany) at 39.5 °C.

As mentioned before, incubations lasted for three different times depending on the incubated substrates: bottles with the 100% F substrate were incubated for 30 h, while those with the 50:50 F:C substrate were incubated for 20 h, and those with the 10:90 F:C substrate were incubated for only 8 h.

Incubations were repeated in 3 runs over 3 different days. The 3 runs were taken as replicates for the statistical analysis. For each treatment, 6 bottles per batch were incubated: the first two bottles were used to assess ruminal fermentation parameters (gas production, NH_3 and lactate concentration, and VFA production) and DMD. Another two bottles were used to measure NDFD and then estimate *in vitro* true substrate digestibility (ivTSD), while the two remaining bottles were used to examine the ruminal digesta FA composition (i.e., the BH process). Moreover, blanks (bottles without substrate and fats) were included to correct for gas production and disappearances, whereas bottles of time 0 were included to correct for VFA. Consequently, the total number of bottles used in this experiment was 204 bottles distributed as follows: [(9 treatments × 3 sets (fermentation, NDF disappearance, and BH) × 2 bottles + 12 blanks (6 for fermentation and 6 for NDF disappearance sets) bottles/run) + 2 bottles of time 0] = 68 bottles/run × 3 runs.

IV.4. PROCEDURES

Gas production was recorded after 2, 5, 8, 12, 20, and 30 h of incubation for the 100% F substrate; 2, 5, 8, 12, and 20 h for the 50:50 F:C substrate; and 2, 5, and 8 h for the 10:90 F:C substrate. A pressure transducer (Gems Sensors 2200, Hampshire, USA) connected to a monitor (Tracker 223; Data Tracker Process Instruments, Birmingham, UK) was used to measure the pressure in psi by puncturing the rubber stopper of each bottle with a needle (0.6 mm in diameter; Sterican B. Braun, Barcelona, Spain) connected to the transducer. After gas measurement, the gas was released, and each bottle was shaken and returned to the incubator until the next gas recording. Pressure values were corrected based on the

exact amount of incubated OM and the gas produced by the blanks. A prediction equation derived from numerous simultaneous gas pressure and volume measurements was used to estimate the gas volume (Hervás et al., 2005).

The production of CO_2 and CH_4 was estimated using the stoichiometric equations described by Groot et al. (1998):

Estimated CO_2 (mmol) = acetate/2 + propionate/4 + 1.5 butyrate.

Estimated CH_4 (mmol) = acetate + 2 butyrate − CO_2.

After 8 (for the 10:90 F:C substrate), 20 (for the 50:50 F:C substrate), and 30 (for the 100% F substrate) h of incubation, the reaction was stopped by placing the bottles in ice-water. Eight mL from each fermentation bottle was transferred to propylene tubes and centrifuged at 3,000 rpm for 10 minutes at 4 °C (Eppendorf 5415C, Madrid, Spain) to remove suspended solids. Then, 0.8 mL of the supernatant of each sample (plus the supernatant from the time 0 bottles) were added to a 1.5 mL Eppendorf tube containing 0.5 mL of a deproteinising solution (20 g/L metaphosphoric acid and 4 g/L crotonic acid in 0.5 M HCl) to measure the VFA concentration. Moreover, 4 mL of the supernatant were mixed with 4 mL of 0.2 N HCl for ammonia concentration analysis. Samples for lactate, VFA, and NH_3 determinations were stored at -30 °C until the subsequent analysis.

The residual content of each fermentation bottle was filtered in previously weighed nylon bags (10 × 20 cm; R1020, Ankom® Technology Corp., Macedon, NY, USA) to measure DMD. Bags were dried in a forced-air oven at 48 °C for 72 h, then weighed again to calculate the DMD.

Bottles for NDF disappearance were stored at -30 °C and then lyophilised. Afterwards, the residues were treated with NDF solution, filtered, and dried. The NDFD was calculated based on the initial incubated NDF content. The ivTSD was estimated using the following equation:

Estimated ivTSD = $(DM_{incubated} - (NDF_{residue} - Blank_{correction}))/DM_{incubated}$

where ivTSD = *in vitro* true substrate digestibility (g/g DM), DM = dry matter (g) and NDF = neutral detergent fibre (g).

To analyse the FA composition of the ruminal digesta (i.e., to assess BH), the contents of the two bottles from the same treatment and run were combined, stored at -80 °C, freeze-dried, homogenised and stored again at -80 °C until further analysis of fatty acids.

IV.5. CHEMICAL ANALYSES

Chemical composition of the feeds

These analyses were conducted in the laboratories of the Department of Nutrition and Production of Herbivores of the IGM, which are certified by SGS (File Certification Number ES20/208722) based on the application rule for ISO 9001:2015.

For DM, samples were oven-dried at 50 °C for 48 h (ISO 6496:1999). Then, dry samples were burned for 6 h at 550 °C in a muffle furnace (12-PR/400; Hobersal, Barcelona, Spain) to assess the ash content (ISO 5984:2002).

Regarding CP analyses, a Kjeldahl autoanalyst (Foss Kjeltec ™ 2400, Foss Iberia, Barcelona, Spain) was used to measure the nitrogen content using potassium sulphate and cupric sulphate as catalysts (ISO 5983-2:2009). The N value was multiplied by the conversion factor 6.25 (CP = N × 6.25) to obtain the CP values.

For fibre fraction determination, NDF, ADF, and acid detergent lignin (ADL) were measured using an Ankom 2000 analyser (Ankom Technology Corp., Macedon, NY, USA), according to the methodology described by Ankom (https://ankom.com; Ankom Technology Methods 13 and 12, respectively). The former was assayed with sodium sulphite and α-amylase and both NDF and ADF were expressed with residual ash.

Finally, the EE content was estimated using the Ankom system (Ankom Filter Bag Technology) and the technique described by the AOCS (2008; Procedure Am 5-04).

Lactate

Samples were analysed colourimetrically using a spectrophotometer at 340 nm and an enzymatic kit (Megazome, Bray Business Park, Bray, Co. Wicklow, A98YV29, Ireland) according to Gawehn (1988) and Noll (1988).

Ammonia and volatile fatty acids

Samples for ammonia and VFA were thawed at 4 °C for 24 h and then centrifuged (3,000 and 10,000 rpm, respectively; 10 minutes, 4 °C). Ammonia concentration was determined colourimetrically using the salicylate method described by Reardon et al. (1966). Concentration of VFA was determined by gas chromatography (GC) using crotonic acid as an internal standard (Ottenstein & Bartley, 1971).

Fatty acid analysis

Fatty acid methyl esters (FAME) of feeds were prepared in a 1-step extraction-transesterification procedure using chloroform and 2% (vol/vol) sulfuric acid in methanol (Shingfield et al., 2003). Tridecenoic acid (Product No. 10-1301; Larodan Fine Chemicals, Sweden) was used as an internal standard.

Fatty acid methyl esters of palm distillate fatty acids, soybean oil, and *T. molitor* oil were prepared using approximately 50 mg in 2 mL of hexane and a base-acid catalysed transesterification procedure (Shingfield et al., 2003).

The lipid content of 200 mg freeze-dried ruminal digesta samples was extracted in duplicate using 4 mL of a hexane and propanol mixture (3:2, v/v) after modifying the pH of the digesta to 2 with 2 M hydrochloric acid. Organic extracts from each sample were mixed and dried at 50 °C under N_2 flow. Then, using a base-acid catalysed transesterification procedure with fresh 0.5 M sodium methoxide prepared in methanol at 20 °C for 5 minutes, the dissolved lipids in 2 mL of hexane were converted to FAME, followed by a reaction with a 1% (vol/vol) sulfuric acid solution in methanol for 30 minutes at 50 °C (Shingfield et al., 2003).

The determination of FAME in feeds, oils, and rumen digesta samples was carried out using a gas chromatograph (Agilent 6890A Network System, USA) equipped with an automatic injector, a flame ionisation detector, and a 100 m fused-silica capillary column (0.25 mm i.d., 0.2 µm film thickness; CP-SIL 88, CP7489; Varian Ibérica SA, Spain), and using hydrogen as a carrier and fuel gas (207 kPa, 2.1 mL/minute). A temperature gradient programme was used to quantify the total FAME in a 0.2 µL sample volume injected with a split ratio of 1:50 (Shingfield et al., 2003).

The identification of the chromatographic peaks of FAME was carried out by comparisons with commercial mixtures of authentic standards (from Nu-Chek Prep., Elysian, MN, USA; Sigma Aldrich Madrid, Spain; and Larodan Fine Chemicals AB, Malmö, Sweden) and with reference samples that had previously been confirmed by gas chromatography and mass spectrometry of the 4,4 dimethyloxazolinic derivatives (Toral et al., 2010c).

IV.6. STATISTICAL ANALYSES

All data of ruminal fermentation, degradation and biohydrogenation were analysed by one-way ANOVA using the MIXED procedure of the SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA). As already explained, analyses were carried out separately for each substrate, and the model included the fixed effect of the lipid supplement and the random effect of the run. Means were adjusted for multiple comparisons using the Bonferroni test. Differences were considered significant at $P < 0.05$ and a tendency towards significance at $0.05 \leq P < 0.10$.

The model used was as follows:

$$Y_{ij}=\mu+S_i+R_j+\epsilon_{ij}$$

where: Y_{ij}, is the response variable; μ, the overall mean of the response variable; S_i, the fixed effect of the lipid supplement; R_j, the random effect of the run, and ϵ_{ij}, the residual error.

V. RESULTS AND DISCUSSION

V.1. CHEMICAL COMPOSITION OF INCUBATED SUBSTRATES

Table 4 shows the chemical composition of the three incubated substrates. As expected, substrates with high levels of concentrate had high contents of CP (18.3 and 18.7% DM in 10:90 and 50:50 F:C, respectively). Conversely, their content in fibre, both NDF and ADF, was relatively low. On the contrary, the 100% F substrate showed a moderate content in CP (12% DM) but high in NDF, ADF and ADL (47.8, 34.6, and 6.86% DM, respectively). The contents of organic matter, ether extract, and ashes seem similar in the three tested substrates.

The 100% F substrate used in this *in vitro* study could be considered as medium-quality forage according to hay classification standards based on its CP and fibre content (Cherney & Parsons, 2020). Due to its high NDF content, the fermentation of this substrate would proceed more slowly (Getachew et al., 2004; Maccarana et al., 2016), which is the reason behind the longer incubation. Its lignin content (6.86% DM) may further limit its fermentability and so the production of fermentation end products (McDonald et al., 2011; Van Soest, 1994). On the other hand, diets with concentrate (10:90 F:C and 50:50 F:C) were expected to show a higher rumen fermentation (Getachew et al., 2004; Van Soest, 1994).

Table 4. Chemical composition (% DM) of *in vitro* substrates.

Item[1]	*In vitro* substrates		
	100% F	50:50 F:C	10:90 F:C
OM	89.2	91.5	91.3
CP	12.0	18.7	18.3
EE	2.83	2.21	3.01
NDF	47.8	30.4	24.9
ADF	34.6	20.5	12.8
ADL	6.86	4.04	2.45
Ash	9.75	8.12	7.96

[1] OM = organic matter, CP = crude protein, EE = ether extract, NDF = neutral detergent fibre, ADF = acid detergent fibre, ADL = acid detergent lignin.

V.2. FATTY ACID COMPOSITION OF SUBSTRATES AND SUPPLEMENTAL FATS

The FA composition of the substrates and the lipid supplements used for the *in vitro* incubation is shown in Table 5. The 100% F substrate was characterised by the high concentrations of 18:3n-3, 16:0, and 18:2n-6. These results are consistent with De Beni Arrigoni et al. (2016) and Glasser et al. (2013), who reported that these FA are predominant in forages. The total FA content of the 100% F substrate was 15.4 mg/g DM. Concerning the 50:50 F:C substrate, it was mainly composed of 18:2n-6, 16:0, *cis*-9 18:1, and 18:3n-3, which is in agreement with many other previous works (e.g., Benchaar et al., 2012; Gómez-Cortés et al., 2011a; Hervás et al., 2022). The 50:50 F:C substrate of this study had a total FA content of 17.1 mg/g DM. Finally, the main FA in the 10:90 F:C substrate was 18:2n-6, followed by *cis*-9 18:1 and 16:0. As stated by Aurousseau et al. (2004) and De Beni Arrigoni et al. (2016), 18:2n-6 and *cis*-9 18:1 are very often the most abundant FA of the lipid fraction in concentrate diets. The 10:90 F:C substrate had a high FA content (i.e., 28.3 mg/g DM) due to the high level of cereals in this substrate (Sauvant et al., 2004; Vlaeminck et al., 2006b).

Regarding the lipids used in this *in vitro* trial, as expected, the palm distillate FA (PFA) was mostly composed of SFA, with the 16:0 accounting for more than 90% of the total FA. The second most abundant FA was the 18:0, present at 8.58% of the total FA. The SBO contained 18:2n-6 as the main FA (about 55% of the total FA), followed by *cis*-9 18:1, 16:0, and 18:3n-3. These results were similar to those obtained in Ozawa et al. (2001) and Sauvant et al. (2004), where 18:2n-6 represented 53.2% of the total FA in SBO. As for the oil extracted from Tenebrio (TMO), the *cis*-9 18:1 was the major FA (about 39% of the total FA content), followed by 18:2n-6 (almost 30%), and 16:0 (around 17%). This FA profile was within the ranges reported in the literature and summarised in the Literature Review (see Table 1; Noyens et al., 2023; Son et al., 2020; Wu et al., 2020). According to that information, the main FA of *T. molitor* oil can be either *cis*-9 *cis*-12 18:2 or *cis*-9 18:1.

Table 5. Fatty acid (FA) composition of the substrates and the fats used in the *in vitro* incubations.

	In vitro substrates			Supplemental fats[1]		
	100% F	50:50 F:C	10:90 F:C	PFA	SBO	TMO
FA profile, g/100 total FA						
12:0	0.525	0.294	0.046	0.029	0.005	0.371
14:0	2.95	1.62	0.955	0.676	0.082	3.76
16:0	26.4	26.0	18.8	90.2	10.8	17.0
cis-9 16:1	0.397	0.330	0.200	0.104	0.085	1.74
17:0	0.607	0.492	0.226	0.062	0.098	0.126
18:0	3.81	4.09	3.04	8.58	4.11	2.64
cis-9 18:1	6.52	14.7	26.1	0.155	19.6	39.1
cis-11 18:1	0.672	1.15	1.85	0.004	2.16	1.66
cis-9 *cis*-12 18:2	24.6	38.0	43.7	0.014	54.7	29.7
cis-9 *cis*-12 *cis*-15 18:3n-3[2]	28.1	10.4	3.65	-	7.38	1.14
20:0	1.36	0.932	0.493	0.101	0.319	0.106
22:0	1.84	0.924	0.468	-	0.335	0.032
24:0	1.82	0.993	0.403	-	0.139	-
Total FA[3]	15.4	17.1	28.3	-	-	-

[1] PFA = palm distillate fatty acids, SBO = soybean oil, TMO = *Tenebrio molitor* oil.

[2] Coelutes with *cis*-11 20:1.

[3] mg FA/g DM.

V.3. *IN VITRO* RUMINAL FERMENTATION

V.3.1. 100% F substrate

Results of ruminal fermentation parameters obtained when the 100% F substrate was incubated for 30 h are presented in Table 6. The PFA treatment had a higher gas production than SBO ($P < 0.05$), while TMO produced an intermediate amount ($P > 0.10$). This lower gas production obtained in the SBO group could be linked to the high content of C18 PUFA in this lipid supplement (more than 60% of total FA vs. 0.014% in PFA and 30.8% in TMO) which have antimicrobial effects against rumen microbiota (De Beni Arrigoni et al., 2016; Nagaraja et al., 1997). Although no significant differences in *in vitro* gas production due to *T. molitor* oil supplementation were found in this study, some studies have linked insect oil to reductions in this parameter, depending on the doses, FA profiles of the insect oils and substrates incubated. Thus, Jayanegara et al. (2020) reported no significant effect on *in vitro* total gas production when *T. molitor* oil was supplemented at 5% DM to a 70:30 F:C

substrate compared to the unsupplemented group. Conversely, Thirumalaisamy et al. (2020) observed a significant reduction in gas production when using silkworm pupae oil at 4% and 5% DM in a 70:30 F:C substrate.

As well known, VFA are the main energy sources for ruminants (Guilloteau et al., 2010; McDonald et al., 2011; Nagaraja, 2016). Besides, VFA are primarily produced by microbial fermentation in the rumen and, therefore, they can be used as indicators of changes in rumen fermentation patterns (An et al., 2024; Owens & Basalan, 2016). In the current study, no differences in total VFA production were found among supplemental fats ($P > 0.10$). Palm FA produced a higher percentage of iso-butyrate than SBO ($P < 0.05$), while intermediate values were obtained in TMO ($P > 0.10$). The increased iso-butyrate content observed in the rumen digesta of PFA compared to SBO could be indicative of enhanced microbial growth of some bacteria populations (An et al., 2023; Roman-Garcia et al., 2021) as iso-VFA are essential growth factors that support the development of numerous ruminal fibre-degrading bacteria (Andries et al., 1987; Owens & Basalan, 2016; Roman-Garcia et al., 2021).

The lipid supplementation to the 100% F substrate did not affect any of the other studied fermentation parameters (i.e., ammonia and lactic acid concentrations, estimates of CO_2 and CH_4 production, A/P ratio, and the rest of the individual VFA; $P > 0.10$). Similarly, no effect of this fat supplementation was found in terms of DMD, NDFD and ivTSD ($P > 0.10$). The results concerning ammonia concentrations are consistent with those of Jayanegara et al. (2020), who stated that dietary supplementation with *T. molitor* oil at 5% DM to a 70:30 F:C substrate had no effect on *in vitro* ammonia concentrations compared to the untreated group. In general, the absence of differences in fermentation parameters could be attributed to the low inclusion level (i.e., 2% DM). However, the addition of a similar level of silkworm pupae oil (2% DM) to a high forage substrate (i.e., 70:30 F:C ratio) was shown to decrease *in vitro* gas production and DMD (Thirumalaisamy et al., 2020), probably due to the high UFA content in silkworm pupae oil (more than 65% of total FA; Chieco et al., 2019; Hu et al., 2017). Moreover, higher levels of supplementation of insect oil (i.e., 5% DM) from *T. molitor* and crickets (Jayanegara et al., 2020) or silkworms (Jayanegara et al., 2020;

Thirumalaisamy et al., 2020) to high forage substrates (70:30 F:C ratio) have also been reported to decrease *in vitro* DM and OM disappearance. These reductions have been attributable to the negative effects of the high UFA content of these oils on the proliferation and activity of rumen microbiota involved in nutrient degradation (De Beni Arrigoni et al., 2016; Jenkins, 1993).

To our knowledge, no previous studies have evaluated the effects of *T. molitor* oil on the fermentation process of an exclusively forage-based diet. Therefore, in view of these initial results, the effect of this TMO on *in vitro* ruminal fermentation parameters indicates that this oil may be an alternative energy source to be considered.

Table 6. Gas production, ammonia and lactic acid concentrations, estimated CO_2 and CH_4 productions, dry matter disappearance (DMD), neutral detergent fibre disappearance (NDFD), *in vitro* true substrate digestibility (ivTSD), total volatile fatty acids (VFA) production and molar proportions of individual VFA, and acetate/propionate (A/P) ratio after 30 h incubation of the 100% F substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	100% F substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
Gas production, mL/g OM	197[a]	188[b]	191[ab]	3.5	0.029
Ammonia, mg/L	245	248	252	5.3	0.582
Lactic acid, mg/L	2.81	2.78	2.91	0.549	0.841
Estimated CO_2 production, mmol	1.04	1.06	1.08	0.025	0.222
Estimated CH_4 production, mmol	0.633	0.643	0.657	0.0129	0.215
DMD, g/g	0.489	0.507	0.518	0.0223	0.363
NDFD, g/g	0.302	0.312	0.307	0.0111	0.826
ivTSD, g/g	0.666	0.671	0.669	0.0053	0.825
Total VFA, mmol/L	49.4	50.6	51.5	1.10	0.115
A/P ratio	2.99	2.97	2.98	0.087	0.866
Molar proportions of VFA (mmol/100 mmol)					
Acetate	65.1	65.3	65.1	0.33	0.354
Propionate	21.8	22.0	21.9	0.53	0.746
Iso-butyrate	0.595[a]	0.560[b]	0.587[ab]	0.0294	0.025
Butyrate	9.68	9.41	9.63	0.163	0.495
Iso-valerate	0.981	0.913	0.972	0.0356	0.074
Valerate	1.71	1.72	1.72	0.104	0.912
Caproate	0.094	0.119	0.124	0.0782	0.703
Iso-acids	1.58	1.47	1.56	0.060	0.039[*]
Minor VFA[4]	3.38	3.31	3.40	0.237	0.280

[1] 100% F substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).

[2] SED = standard error of the difference.

[3] Probability of significant effects of the experimental treatment.

[4] Calculated as the sum of percentages of isobutyrate, isovalerate, valerate, and caproate acids.

[*] Differences were not significant after using the Bonferroni test.

[a-b] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.3.2. 50:50 F:C substrate

As reported in Table 7, *in vitro* incubation of the 50:50 F:C substrate for 20 h showed no significant differences among treatments for any of the studied fermentation parameters, regardless of the supplemental fats used ($P > 0.10$).

As mentioned before for the 100% F substrate, the lack of effect in gas production among treatments in the 50:50 F:C substrate could be attributed to the low inclusion level of lipid supplements (2% DM). It may also be related to the relatively short incubation period (20 h), reflecting the rumen retention time. The results of the present study are consistent with those of Hervás et al. (2022), who observed no significant difference in *in vitro* gas production when a 55:45 F:C TMR was supplemented with the same inclusion level (2% DM) of house cricket, black soldier fly, or silkworm oils for 16 h. Nevertheless, when higher rates of inclusion (4% and 5% DM) of silkworm pupae oil were added in a 50:50 F:C substrate in Thirumalaisamy et al. (2020), a decrease in gas production attributed to the insect oil was observed. This was linked to the negative effects of high lipid content on ruminal fermentation. Notably, although the incubation time was greater in the 100% F substrate, the DMD, NDFD and ivTSD were higher in the 50:50 F:C substrate, probably reflecting the lower fermentation and degradation of the 100% F substrate due to its higher fibre content (Getachew et al., 2004). The greater degradation observed in the 50:50 F:C substrate could be related to its relatively lower ADL fraction compared to the 100% F substrate (4.04 and 6.86% DM, respectively), as lignin is known to limit cellulose degradation in the rumen (McDonald et al., 2011; Van Soest, 1994). In addition, the lower NDF content of the 50:50 F:C substrate may also be a reason for the enhanced DMD and ivTSD observed in this study, as high NDF levels are known to affect fermentation efficiency in the rumen (Maccarana et al., 2016; Shi et al., 2023).

The similar results obtained for VFA and ammonia among lipid treatments may reflect no differences concerning energy and protein ruminal metabolism. In any event, the lack of differences in ruminal parameters in TMO supplementation compared to the other two conventional sources (i.e., PFA and SBO) in the 50:50 F:C substrate is a promising result for using this insect oil in the diet of dairy sheep. This conclusion was previously stated by Hervás et al. (2022). Since the addition of higher levels of silkworm pupae oil (5% DM) to a 50:50 F:C substrate was reported to decrease DMD *in vitro* (Thirumalaisamy et al., 2020), higher TMO inclusions could lead to detrimental effects concerning ruminal degradation.

Table 7. Gas production, ammonia and lactic acid concentrations, estimated CO_2 and CH_4 productions, dry matter disappearance (DMD), neutral detergent fibre disappearance (NDFD), *in vitro* true substrate digestibility (ivTSD), total volatile fatty acids (VFA) production and molar proportions of individual VFA, and acetate/propionate (A/P) ratio after 20 h incubation of the 50:50 F:C substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	50:50 F:C substrate[1]				
	+2% PFA	+2% SBO	+2% TMO	SED[2]	*P*-value[3]
Gas production, mL/g OM	193	191	191	3.1	0.645
Ammonia, mg/L	243	236	236	5.4	0.303
Lactic acid, mg/L	1.81	2.45	2.07	0.308	0.099
Estimated CO_2 production, mmol	1.17	1.20	1.17	0.020	0.515
Estimated CH_4 production, mmol	0.613	0.633	0.613	0.0226	0.831
DMD, g/g	0.646	0.644	0.655	0.0186	0.670
NDFD, g/g	0.384	0.359	0.380	0.0107	0.269
ivTSD, g/g	0.817	0.812	0.812	0.0064	0.829
Total VFA, mmol/L	51.9	53.1	52.0	0.97	0.603
A/P ratio	2.32	2.36	2.31	0.151	0.269
Molar proportions of VFA (mmol/100 mmol)					
Acetate	57.2	57.5	57.1	0.96	0.063
Propionate	24.7	24.5	24.9	1.16	0.392
Isobutyrate	0.544	0.535	0.519	0.0566	0.562
Butyrate	14.3	14.3	14.4	0.31	0.684
Isovalerate	0.867	0.855	0.833	0.0764	0.547
Valerate	2.17	2.16	2.19	0.124	0.068
Caproate	0.099	0.089	0.078	0.0799	0.251
Iso-acids	1.41	1.39	1.35	0.131	0.521
Minor VFA[4]	3.68	3.63	3.62	0.335	0.599

[1] 50:50 F:C substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Calculated as the sum of percentages of isobutyrate, isovalerate, valerate, and caproate acids.

V.3.3. 10:90 F:C substrate

Table 8 reports the results of ruminal fermentation parameters obtained when the 10:90 F:C substrate was incubated for 8 h. Lactic acid concentrations were higher in the palm FA than in the SBO treatment ($P < 0.05$), while TMO values did not differ from any of the above ($P > 0.10$). The greater lactic acid concentrations obtained with the palm FA treatment could be due to the FA composition of this lipid supplement, mostly composed

by SFA, which can promote the growth of lactic acid-producing bacteria (Maia et al., 2007). In addition, SBO led to the highest proportion of butyrate ($P < 0.05$), while palm FA and *T. molitor* oil had similar percentages ($P > 0.10$). If this could be translated to *in vivo* conditions, the increased butyrate proportion in the SBO group could be associated with improved epithelial cell development and so rumen surface area available for absorption (Guilloteau et al., 2010; Mariadason et al., 1999; McDonald et al., 2011). Although high dietary PUFA levels have been reported to reduce butyrate concentrations in rumen digesta (Jian et al., 2016; Patra, 2013) likely due to the inhibition of fibrolytic bacterial activity (Jian et al., 2016; Maia et al., 2007; Nagaraja et al., 1997), this effect was not observed in the present study. In contrast, SBO, which had the highest PUFA content, was associated with the greatest butyrate proportion. In the rest of the studied fermentation parameters, no significant difference was observed among treatments ($P > 0.10$). The similar values in estimated CO_2 and CH_4 production could be attributed to the low level of lipid supplementation, since a higher inclusion level of *T. molitor* oil (5% DM) was found to decrease methane emissions *in vitro* (Jayanegara et al., 2020). Similarly, Thirumalaisamy et al. (2020) reported a significant decrease in methane production with the addition of silkworm pupae oil at 4% and 5% DM, both in diets with similar F:C ratios. The reduced methane emissions in these studies were probably linked to a decline in the abundance or activity of methanogenic bacteria and a reduction in nutrient degradation (Guyader et al., 2015; Nagaraja et al., 1997; Jayanegara et al., 2020).

Without any intention to compare the 3 substrates, which would not be correct, it may be interesting to note that the relatively low total gas production in this highly concentrated substrate (10:90 F:C) could arise from its low fibre content (Getachew et al., 1998; Russell, 1998). The high proportion of concentrate may also be related to the relatively lower rumen ammonia (Agle et al., 2010; Hristov et al., 2005) and higher lactic acid (Ewaschuk et al., 2005; Møller et al., 1997) concentrations.

Table 8. Gas production, ammonia and lactic acid concentrations, estimated CO_2 and CH_4 productions, dry matter disappearance (DMD), neutral detergent fibre disappearance (NDFD), *in vitro* true substrate digestibility (ivTSD), total volatile fatty acids (VFA) production and molar proportions of individual VFA, and acetate/propionate (A/P) ratio after 8 h incubation of the 10:90 F:C substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	10:90 F:C substrate[1]				
	+2% PFA	+2% SBO	+2% TMO	SED[2]	*P*-value[3]
Gas production, mL/g OM	108	104	102	3.4	0.150
Ammonia, mg/L	153	150	153	9.8	0.410
Lactic acid, mg/L	551[a]	498[b]	524[ab]	106.8	0.008
Estimated CO_2 production, mmol	0.697	0.710	0.700	0.0272	0.596
Estimated CH_4 production, mmol	0.363	0.367	0.363	0.0233	0.934
DMD, g/g	0.494	0.486	0.497	0.0235	0.594
NDFD, g/g	0.067	0.073	0.055	0.0095	0.595
ivTSD, g/g	0.768	0.771	0.773	0.0055	0.712
Total VFA, mmol/L	30.9	31.4	31.3	1.17	0.701
A/P ratio	2.22	2.19	2.16	0.128	0.430
Molar proportions of VFA (mmol/100 mmol)					
Acetate	58.9	58.4	58.5	1.17	0.322
Propionate	26.6	26.8	27.2	1.13	0.392
Isobutyrate	0.068	0.065	0.044	0.0310	0.334
Butyrate	13.4[b]	13.8[a]	13.2[b]	0.32	0.008
Isovalerate	0.156	0.129	0.133	0.0293	0.332
Valerate	0.914	0.827	0.903	0.1608	0.239
Caproate	0.004	0.000	0.017	0.0056	0.154
Iso-acids	0.215	0.186	0.177	0.0603	0.516
Minor VFA[4]	1.13	0.99	1.08	0.197	0.251

[1] 10:90 F:C substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).

[2] SED = standard error of the difference.

[3] Probability of significant effects of the experimental treatment.

[4] Calculated as the sum of percentages of isobutyrate, isovalerate, valerate, and caproate acids.

[a-b] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4. RUMINAL BIOHYDROGENATION OF FATTY ACIDS

Results on ruminal digesta FA composition show a number of effects of lipid supplementation in each of the three incubated substrates. Although we are not discussing each and every variation, we consider of utmost importance to report a comprehensive FA profile (58 compounds). As noted in a recent invited review by researchers from the team in which the author of this MSc memory was integrated (Toral et al., 2024): "Reporting FA profiles as completely as possible is key not only to provide information that can be useful in the future (if the activity of an FA is discovered in the future), but also to evaluate the quality of the analysis (such as the accuracy of FA identifications and the presence of coelutions and possible analytical artifacts)". In addition, reporting such a comprehensive profile may be a bedrock for future research on the use of insects or insect products in ruminants, due to the potential impact of some BH intermediates with biological activity.

V.4.1. 100% F substrate

V.4.1.1. Saturated FA

Table 9 reports the profile of SFA in the ruminal digesta of the 100% F substrate.

First of all, it is worth remembering that the accumulation of SFA in the rumen digesta depends on their dietary uptake and/or on the activity of ruminal microorganisms through BH of UFA (Buccioni et al., 2012; Shingfield et al., 2013; Toral et al., 2018). Starting with even-chain SFA, the supplementation with *T. molitor* oil sharply decreased the proportion of 16:0 compared to PFA, and the lowest percentage was found with SBO ($P < 0.05$). The remarkable higher content in 16:0 and, consequently, in total SFA content ($P < 0.05$) with PFA supplementation was most probably due to the high dietary supply of 16:0 provided by palm distillate FA. Conversely, the percentages of 16:0 found in the rumen digesta of SBO and TMO may reflect their lower dietary 16:0 levels. The percentage of 18:0 found in TMO was similar to that obtained in SBO and both were higher than in PFA treatment ($P < 0.05$). Since the 18:0 is the end product of ruminal BH of C18 unsaturated FA (Griinari & Bauman, 1999; Harfoot & Hazlewood, 1997; Toral et al., 2018), its greater levels in SBO and TMO could be a consequence of the BH of the higher proportions of C18 UFA provided by these

lipids. Besides, *T. molitor* oil treatment resulted in greater percentages of 10-oxo-18:0 compared to the other treatments ($P < 0.05$). The high content of *cis*-9 18:1 in the TMO used in the current study can explain this increase, as 10-oxo-18:0 is mainly produced from *cis*-9 18:1 hydrogenation (Jenkins et al., 2006; Márquez-Ruiz et al., 2011). This greater 10-oxo-18:0 content may suggest that *T. molitor* oil favoured the proliferation or activity of bacterial populations involved in ruminal FA hydration and oxidation reactions responsible for the production of this FA (Hudson et al., 2000; Jenkins et al., 2006).

Concerning ruminal odd-chain FA (OCFA) and branched-chain FA (BCFA), they are largely produced by rumen bacteria and, therefore, they have been proposed as biomarkers of ruminal microbiota and fermentation function (Fievez et al., 2012; Vlaeminck et al., 2006a). In our study, the supplementation with TMO promoted the concentrations of several OCFA (such as 15:0, 17:0, and 19:0) and some BCFA (such as 16:0 *iso* and 17:0 *anteiso*), compared to the other treatments ($P < 0.05$). Total OCFA in TMO was statistically similar to that in SBO ($P > 0.10$), whereas total BCFA was significantly higher in TMO than in the other treatments ($P < 0.05$). The greater proportions of several OCFA and BCFA in the rumen digesta of TMO can suggest beneficial effects of this insect oil on the rumen microbiota responsible for the synthesis of these FA, such as fibre-degrading bacteria (Fievez et al., 2012; Vlaeminck et al., 2006a, b). Increasing the ruminal flux of OCFA and BCFA would increase the milk fat content of these FA, given that milk OCFA and BCFA are greatly derived from the bacteria escaping from the rumen (Toral et al., 2020; Vlaeminck et al., 2006a). This is a desirable finding from a consumer health perspective, as BCFA are bioactive FA with potential benefits in humans (Ran-Ressler et al., 2014; Shingfield et al., 2008).

Table 9. Concentration (g/100 g FA) of saturated fatty acids of the ruminal digesta after 30 h incubation of the 100% F substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	100% F substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
12:0	0.193	0.180	0.211	0.0115	0.127
13:0 *iso*	0.084	0.083	0.085	0.0059	0.967
13:0	0.089^{b}	0.095^{ab}	0.103^{a}	0.0033	0.032
14:0 *iso*	0.274	0.256	0.287	0.0105	0.093
14:0[4]	2.44	2.33	3.24	0.2920	0.065
15:0 *iso*	0.175	0.169	0.187	0.0085	0.212
15:0 *anteiso*	0.888	0.855	0.899	0.0536	0.720
15:0	1.09^{c}	1.16^{b}	1.24^{a}	0.021	0.005
16:0 *iso*	0.209^{b}	0.205^{b}	0.217^{a}	0.0027	0.026
16:0	51.9^{a}	16.2^{c}	20.3^{b}	1.17	<0.001
17:0 *iso*	0.142	0.202	0.216	0.0376	0.226
17:0 *anteiso*	0.207^{b}	0.207^{b}	0.250^{a}	0.0101	0.021
17:0	0.414^{c}	0.498^{b}	0.548^{a}	0.0079	<0.001
18:0	20.3^{b}	33.2^{a}	32.8^{a}	0.67	<0.001
10-oxo-18:0[5]	0.399^{c}	0.850^{b}	1.52^{a}	0.023	<0.001
13-oxo-18:0	0.180	0.233	0.228	0.0169	0.062
19:0	0.059^{b}	0.065^{b}	0.085^{a}	0.0057	0.023
20:0	0.552^{c}	0.703^{a}	0.617^{b}	0.0129	<0.001
21:0	0.122^{b}	0.229^{a}	0.148^{b}	0.0119	0.002
22:0[6]	0.543^{c}	0.720^{a}	0.623^{b}	0.0128	<0.001
23:0[7]	0.272	0.298	0.309	0.0110	0.064
24:0	0.418^{b}	0.490^{a}	0.467^{a}	0.0138	0.015
25:0	0.067	0.074	0.071	0.0058	0.503
26:0	0.283^{b}	0.315^{a}	0.321^{a}	0.0097	0.034
27:0	0.029	0.029	0.027	0.0030	0.787
28:0	0.309	0.306	0.338	0.0207	0.327
∑ OCFA[8]	2.14^{b}	2.45^{a}	2.53^{a}	0.032	<0.001
∑ BCFA[9]	2.04^{b}	2.04^{b}	2.22^{a}	0.037	0.012
∑ SFA[10]	81.2^{a}	59.3^{c}	64.8^{b}	1.50	<0.001

[1] 100% F substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with DMA 15:0 *anteiso*.
[5] Coelutes with 9-oxo-18:0 as a minor component.
[6] Coelutes with a contaminant compound.
[7] Coelutes with geraniol.
[8] OCFA = odd-chain FA; calculated as the sum of fatty acids with an odd number of carbon atoms.
[9] BCFA = branched-chain FA; represents the summation of *iso* and *anteiso* fatty acids.
[10] SFA = saturated FA; all fatty acids with no double bonds.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4.1.2. Monounsaturated FA

Results of MUFA concentrations in the ruminal digesta of the 100% F substrate are reported in Table 10. Increasing concentrations of *trans*-11 18:1 were found in TMO and SBO compared to PFA ($P < 0.05$). The high content of this FA in the rumen digesta of SBO and TMO of the 100% F substrate can be attributed to the BH of the higher percentage of C18 UFA in these treatments. This VA is well known as a health-promoting FA (Fan et al., 2023; Wang et al., 2008), and it is the precursor for the endogenous synthesis of RA (which also has beneficial effects for consumers health; Dilzer & Park, 2012; Ip et al., 1995; Lee et al., 1994) in the mammary gland and adipose tissue via the Δ^9-desaturation process (Bauman et al., 1999; Buccioni et al., 2012; Shingfield et al., 2008). Thus, a healthier FA profile of ruminant products could be achieved by the replacement of PFA by TMO, as promoting the flow of *trans*-11 18:1 from the rumen is a target in ruminants' nutrition (Baila et al., 2023; Griinari et al., 2000). In addition, as a consequence of the BH of UFA, TMO also showed higher percentages of *trans*-9 and *trans*-10 18:1 than PFA ($P < 0.05$), with values similar to those obtained in SBO ($P > 0.10$). However, although the *trans*-10 18:1 isomer content was increased in both SBO and *T. molitor* oil treatments, this was not accompanied by a shift towards *trans*-10 18:1 production, as the *trans*-10/*trans*-11 18:1 ratio remained statistically similar among the three groups ($P > 0.10$). A greater *trans*-10 18:1 production has been linked with detrimental productive implications, particularly for being the precursor of *trans*-10 *cis*-12 CLA, which has been associated with milk fat depression (Bauman & Griinari, 2003; Bauman et al., 2003).

Concentrations of *cis*-15 and *trans*-15 18:1 increased from PFA to TMO and SBO ($P < 0.05$). The increased proportion of these FA in the rumen digesta of SBO and TMO could indicate a greater BH of *cis*-9 *cis*-12 *cis*-15 18:3 due to the higher content of this FA in both oils (Ferlay et al., 2017; Jenkins et al., 2008). Moreover, the supplementation of the 100% F substrate with TMO at 2% DM promoted *cis*-9 (+*trans*-13+14) 18:1 percentages compared to the other treatments ($P < 0.05$), which could be explained by the concentration of this FA in TMO. However, it must be mentioned that the BH of *cis*-9 *cis*-12 18:2 and *cis*-9 *cis*-12 *cis*-15 18:3 may also produce *cis*-9 18:1 in the rumen digesta (Kishino et al., 2013; Maia et al.,

2007; Toral et al., 2024). This FA, *cis*-9 18:1, has been shown to produce beneficial health effects in humans (Calder, 2015; Shingfield et al., 2008).

In line with the above-mentioned higher BH of dietary UFA in SBO and TMO, the supplementation with SBO significantly raised total *trans* 18:1 compared to the other treatments, with TMO having higher contents than PFA ($P < 0.05$). The summation of *cis* 18:1 and total MUFA reached their highest values in both TMO and SBO ($P < 0.05$).

Table 10. Concentration (g/100 g FA) of monounsaturated fatty acids of the ruminal digesta after 30 h incubation of the 100% F substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	100% F substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
cis-7 16:1	0.149	0.154	0.250	0.0499	0.188
cis-9 16:1	0.082[b]	0.056[b]	0.202[a]	0.0219	0.005
trans-4 18:1	0.054[c]	0.105[b]	0.127[a]	0.0031	<0.001
trans-5 18:1	0.025[c]	0.058[b]	0.068[a]	0.0036	<0.001
trans-6+7+8 18:1	0.216[c]	0.584[b]	0.622[a]	0.0131	<0.001
trans-9 18:1	0.186[b]	0.452[a]	0.512[a]	0.0301	<0.001
trans-10 18:1	0.274[b]	0.900[a]	0.797[a]	0.0642	0.001
trans-11 18:1	4.02[c]	12.04[a]	9.63[b]	0.362	<0.001
trans-12 18:1	0.362[c]	1.085[a]	0.853[b]	0.0406	<0.001
trans-15 18:1	0.423[c]	0.897[a]	0.850[b]	0.0168	<0.001
trans-16+*cis*-14 18:1[4]	0.311[c]	0.566[a]	0.493[b]	0.0067	<0.001
trans-10/*trans*-11 18:1	0.068	0.075	0.084	0.0092	0.251
∑ *trans* 18:1	5.88[c]	16.7[a]	14.0[b]	0.40	<0.001
cis-9+*trans*-13+14 18:1	4.89[c]	9.02[b]	10.86[a]	0.620	0.002
cis-11 18:1	0.411[c]	0.874[a]	0.588[b]	0.0421	0.001
cis-12 18:1	0.132[c]	0.332[a]	0.238[b]	0.0058	<0.001
cis-13 18:1	0.034[b]	0.055[a]	0.043[ab]	0.0052	0.034
cis-15 18:1	0.112[c]	0.185[a]	0.155[b]	0.0071	0.001
cis-16 18:1	0.066[b]	0.108[a]	0.101[a]	0.0052	0.003
∑ *cis* 18:1	5.64[b]	10.6[a]	12.0[a]	0.66	0.001
∑ 20:1	0.008	0.012	0.011	0.0029	0.429
cis-13 20:1	0.022	0.019	0.020	0.0057	0.923
cis-15 24:1	0.059	0.050	0.060	0.0047	0.168
∑ MUFA[5]	11.8[b]	27.6[a]	26.5[a]	0.94	<0.001

[1] 100% F substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with *trans*-10 *trans*-14 18:2.

[5] MUFA = monounsaturated FA.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4.1.3. Polyunsaturated FA

The concentration of PUFA in the ruminal digesta of the 100% F substrate can be found in Table 11. Compared to PFA, the supplementation with *T. molitor* oil led to greater concentrations of *cis*-9 *cis*-12 18:2, total CLA and total UFA, with the highest levels of these FA being observed in the SBO treatment ($P < 0.05$). The increased proportions of *cis*-9 *cis*-12 18:2 in SBO and TMO could be directly related to its higher percentage in both oils (the concentration in SBO was 1.8-fold greater than in TMO). In line with this, the highest dietary contribution of *cis*-9 *cis*-12 18:2 in SBO led to the greatest *cis*-9 *trans*-11 CLA (RA) percentages, which were also higher in TMO compared to PFA ($P < 0.05$). Rumen concentrations of this CLA are associated with feeding diets and/or lipid supplements rich in *cis*-9 *cis*-12 18:2 (Bauman & Griinari, 2003; Bauman et al., 1999). The increased RA content in the *T. molitor* oil treatment compared to PFA, together with the above-mentioned rise in VA, would support the use of this insect oil as a substitute for PFA to promote milk and meat contents of this bioactive FA (Bauman et al., 1999; Palmquist et al., 2005). The supplementation with SBO also increased *trans*-9 *cis*-11 CLA, *trans*-11 *trans*-13 CLA, and total PUFA compared to PFA and TMO ($P < 0.05$), without differences between these two groups ($P > 0.10$). It also led to greater percentages of *trans*-10 *cis*-12 CLA compared to PFA ($P < 0.05$), with values statistically similar to TMO ($P > 0.10$). The increase in *trans*-9 *cis*-11, *trans*-10 *cis*-12, and *trans*-11 *trans*-13 CLA in the SBO treatment (together with the reported increase in *trans*-10 18:1) could be associated with a higher risk of milk fat depression (Bauman et al., 2003; Perfield et al., 2007; Toral et al., 2019). Moreover, the higher proportions of *trans*-9 *cis*-11 CLA and *trans*-11 *trans*-13 CLA in SBO would also indicate a greater BH of *cis*-9 *cis*-12 18:2 and *cis*-9 *cis*-12 *cis*-15 18:3 in this treatment, as *trans*-9 *cis*-11 CLA and *trans*-11 *trans*-13 CLA are intermediates of the BH of one or both FA (Lee & Jenkins, 2011a, b; Ferlay et al., 2017; Toral et al., 2024).

Table 11. Concentration (g/100 g FA) of polyunsaturated fatty acids of the ruminal digesta after 30 h incubation of the 100% F substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	100% F substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
cis-9 *cis*-12 18:2	1.38[c]	5.86[a]	2.98[b]	0.388	<0.001
cis-11 *cis*-14 18:2	0.016	0.015	0.017	0.0032	0.771
trans-9 *trans*-12 18:2	0.047	0.060	0.063	0.0044	0.052
trans-9 *cis*-12 18:2	0.161	0.100	0.078	0.0783	0.592
trans-11 *cis*-15 18:2[4]	1.028	0.601	0.448	0.5681	0.610
∑ non-conjugated 18:2	2.98[b]	7.05[a]	3.96[b]	0.380	0.001
cis-9 *trans*-11 CLA[5]	0.324[c]	0.826[a]	0.546[b]	0.0661	0.004
trans-9 *cis*-11 CLA	0.115[b]	0.247[a]	0.166[b]	0.0283	0.024
trans-10 *cis*-12 CLA	0.324[b]	0.434[a]	0.373[ab]	0.0225	0.020
trans-11 *trans*-13 CLA	0.067[b]	0.155[a]	0.084[b]	0.0066	<0.001
Other *trans trans* CLA	0.795[c]	1.744[a]	1.171[b]	0.1288	0.005
∑ CLA	1.63[c]	3.41[a]	2.34[b]	0.197	0.002
cis-9 *cis*-12 *cis*-15 18:3[6]	1.57	1.70	1.48	0.100	0.197
trans-9 *trans*-12 *cis*-15 18:3	0.061	0.064	0.071	0.0063	0.380
∑ PUFA[7]	6.36[b]	12.3[a]	8.01[b]	0.676	0.002
∑ UFA[8]	18.2[c]	39.9[a]	34.5[b]	1.49	<0.001

[1] 100% F substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with *trans*-10 *cis*-15 18:2.
[5]Contains *trans*-7 *cis*-9 and *trans*-8 *cis*-10 conjugated linoleic acid (CLA).
[6] Coelutes with *cis*-11 20:1.
[7] PUFA = polyunsaturated FA.
[8] UFA = unsaturated FA.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4.2. 50:50 F:C substrate

V.4.2.1. Saturated FA

The results of SFA in the ruminal digesta of the 50:50 F:C substrate can be found in Table 12. The supplementation with TMO resulted in greater concentrations of 14:0 in the rumen digesta compared to PFA and SBO ($P < 0.05$), which could be attributed to the high proportions of 14:0 in *T. molitor* oil. Notably, this FA (14:0) has been reported to have atherogenic effects (Calder, 2015; German & Dillard, 2004; Shingfield et al., 2008). Furthermore, as observed in the 100% F substrate, the predominance of *cis*-9 18:1 in TMO

could be the cause of the ruminal increase in 10-oxo-18:0 observed with this treatment. Furthermore, 18:0 values in the digesta of TMO were similar to that observed in SBO ($P > 0.10$) and both were higher than in PFA ($P < 0.05$). Conversely, PFA greatly promoted 16:0 levels versus both TMO and SBO, with SBO having the lowest percentage ($P < 0.05$). The changes observed in the levels of 16:0 and 18:0 in the rumen digesta of the 50:50 F:C substrate due to dietary supplementation with PFA, SBO, and *T. molitor* oil were similar to those discussed for the 100% F substrate. Therefore, they will not be discussed further.

Concerning the effect of the lipid supplements on OCFA and BCFA, TMO enhanced the rumen digesta content of 17:0 *anteiso* and 17:0 compared to the other treatments ($P < 0.05$), as previously reported for the 100% F substrate. However, in this case, no differences in total BCFA were found among treatments ($P > 0.10$). In addition, concentrations of 17:0 *iso,* 23:0, and total OCFA in SBO and TMO were higher than in PFA ($P < 0.05$). Once again, the higher proportions of some individual BCFA and OCFA in the rumen digesta of the *T. molitor* oil treatment would indicate beneficial effects of this insect oil on the proliferation and activity of rumen microbiota involved in the *de novo* synthesis of these FA (Fievez et al., 2012; Vlaeminck et al., 2006a, b). Besides, and although we do not intend to compare incubation substrates, it is interesting to mention that the lower proportions of individual and total OCFA in the rumen digesta of this 50:50 F:C substrate could be due to the lower forage fraction, which can favour the proliferation of amylolytic bacteria, decreasing cellulolytic bacteria development. The latter has been found to substantially increase BCFA in the rumen digesta, as they have higher contents of these FA in their membranes (Bas et al., 2003; Vlaeminck et al., 2006a, b).

Table 12. Concentration (g/100 g FA) of saturated fatty acids of the ruminal digesta after 20 h incubation of the 50:50 F:C substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	50:50 F:C substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
12:0	0.112	0.101	0.166	0.0220	0.078
13:0 *iso*	0.069	0.073	0.075	0.0027	0.153
13:0	0.051	0.065	0.072	0.0063	0.068
14:0 *iso*	0.127	0.136	0.135	0.0112	0.705
14:0[4]	1.80[b]	1.79[b]	2.88[a]	0.111	<0.001
15:0 *iso*	0.166	0.174	0.191	0.0125	0.240
15:0 *anteiso*	0.689	0.729	2.090	1.0529	0.404
15:0	0.656	0.695	0.749	0.0270	0.063
16:0 *iso*	0.116	0.134	0.136	0.0119	0.297
16:0	49.6[a]	14.6[c]	18.0[b]	0.57	<0.001
17:0 *iso*	0.165[b]	0.178[a]	0.188[a]	0.0048	0.021
17:0 *anteiso*	0.137[b]	0.153[b]	0.189[a]	0.0121	0.029
17:0	0.382[c]	0.450[b]	0.482[a]	0.0052	<0.001
18:0	21.3[b]	31.1[a]	29.3[a]	1.01	0.001
10-oxo-18:0[5]	0.267[c]	0.552[b]	0.835[a]	0.0669	0.003
13-oxo-18:0	0.138	0.184	0.167	0.0147	0.081
19:0	0.061	0.058	0.074	0.0059	0.111
20:0	0.478[b]	0.596[a]	0.514[b]	0.0132	0.002
21:0	0.092[b]	0.182[a]	0.116[b]	0.0098	0.002
22:0[6]	0.354[c]	0.512[a]	0.433[b]	0.0114	<0.001
23:0[7]	0.188[b]	0.213[a]	0.219[a]	0.0087	0.049
24:0	0.308[b]	0.405[a]	0.379[a]	0.0114	0.002
25:0	0.057	0.071	0.055	0.0072	0.148
26:0	0.229[b]	0.270[a]	0.246[ab]	0.0104	0.040
27:0	0.025	0.021	0.024	0.0048	0.775
28:0	0.143	0.146	0.133	0.0062	0.203
∑ OCFA[8]	1.51[b]	1.76[a]	1.79[a]	0.023	<0.001
∑ BCFA[9]	1.51	1.62	3.06	1.033	0.346
∑ SFA[10]	77.4[a]	53.1[c]	57.5[b]	1.06	<0.001

[1] 50:50 F:C substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with DMA 15:0 *anteiso*.
[5] Coelutes with 9-oxo-18:0 as a minor component.
[6] Coelutes with a contaminant compound.
[7] Coelutes with geraniol.
[8] OCFA = odd-chain FA; calculated as the sum of fatty acids with an odd number of carbon atoms.
[9] BCFA = branched-chain FA; represents the summation of *iso* and *anteiso* fatty acids.
[10] SFA = saturated FA; all fatty acids with no double bonds.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4.2.2. Monounsaturated FA

Table 13 reports the concentrations of MUFA in the ruminal digesta of the 50:50 F:C substrate. Concentrations of several *trans* 18:1 (i.e., *trans*-10, *trans*-11, *trans*-12, *trans*-16+*cis*-14 18:1), *cis*-18:1 (i.e., *cis*-11 and *cis*-12 18:1), and total *trans* 18:1 increased from PFA to TMO and SBO supplementations ($P < 0.05$). Again, the greater UFA proportion in SBO and TMO would be the reason of the increase in those intermediate FA produced during UFA ruminal BH. Dietary supplementation of the 50:50 F:C substrate with the 3 lipids (PFA, SBO and TMO) at 2% DM showed similar effects to those reported for the 100% F substrate regarding *trans*-11 18:1 proportions and *trans*-10/*trans*-11 18:1 ratio. The *in vitro* incubation of a full-fat meal from *T. molitor* as the sole substrate showed similar *trans*-9, *trans*-10 and *trans*-11 18:1 levels in the rumen digesta to those of soybean meal, probably due to the high lipid content of the full-fat insect meal used (Renna et al., 2022a). However, in the current study, *T. molitor* oil reduced *trans*-10 18:1 content compared to SBO ($P < 0.05$). The lower *trans*-10 18:1 in the TMO treatment might be due to the lower *cis*-9 *cis*-12 18:2 content and its potential antimicrobial effects on *trans*-10 18:1-producing microbiota (Zened et al., 2012, 2013). In addition, *trans*-15 18:1 was greater in SBO and TMO compared to PFA ($P < 0.05$), with levels of *cis*-15 18:1 increasing from PFA, to TMO and SBO ($P < 0.05$). As previously explained for the 100% F substrate, these results would be linked to greater BH of *cis*-9 *cis*-12 *cis*-15 18:3 in the latter treatments (Ferlay et al., 2017; Jenkins et al., 2008). Moreover, the highest concentrations of *cis*-9 (+*trans*-13+14) 18:1 and total *cis* 18:1 were observed in TMO, with higher values in SBO than in PFA ($P < 0.05$). As mentioned for the first substrate, these differences in *cis*-9 18:1 may reflect both its contents in the supplements and the variations in the rumen BH of *cis*-9 *cis*-12 18:2 and *cis*-9 *cis*-12 *cis*-15 18:3 (Kishino et al., 2013; Maia et al., 2007; Toral et al., 2024). Finally, *T. molitor* oil greatly increased total MUFA percentages compared to PFA ($P < 0.05$), with no differences with SBO ($P > 0.10$). This was expected due to the marked increase in total *cis* and *trans* 18:1 isomers in these treatments.

Table 13. Concentration (g/100 g FA) of monounsaturated fatty acids of the ruminal digesta after 20 h incubation of the 50:50 F:C substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	50:50 F:C substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
cis-7 16:1	0.112[b]	0.125[b]	0.296[a]	0.0250	0.003
cis-9 16:1	0.064[b]	0.060[b]	0.237[a]	0.0159	<0.001
trans-4 18:1	0.072[c]	0.158[b]	0.173[a]	0.0035	<0.001
trans-5 18:1	0.044[b]	0.108[a]	0.118[a]	0.0045	<0.001
trans-6+7+8 18:1	0.383[b]	0.955[a]	0.945[a]	0.0209	<0.001
trans-9 18:1	0.322[c]	0.617[b]	0.705[a]	0.0180	<0.001
trans-10 18:1	0.624[c]	1.515[a]	1.184[b]	0.0272	<0.001
trans-11 18:1	5.46[c]	13.43[a]	10.43[b]	0.391	<0.001
trans-12 18:1	0.553[c]	1.336[a]	1.070[b]	0.0261	<0.001
trans-15 18:1	0.433[b]	0.935[a]	0.967[a]	0.0388	<0.001
trans-16+*cis*-14 18:1[4]	0.291[c]	0.535[a]	0.430[b]	0.0133	<0.001
trans-10/*trans*-11 18:1	0.115	0.114	0.114	0.0075	0.988
∑ *trans* 18:1	8.18[c]	19.58[a]	16.02[b]	0.411	<0.001
cis-9+*trans*-13+14 18:1	6.21[c]	11.02[b]	14.10[a]	0.259	<0.001
cis-11 18:1	0.562[c]	1.090[a]	0.855[b]	0.0236	<0.001
cis-12 18:1	0.188[c]	0.419[a]	0.299[b]	0.0122	<0.001
cis-13 18:1	0.040[b]	0.065[a]	0.047[ab]	0.0066	0.048
cis-15 18:1	0.078[b]	0.122[a]	0.096[b]	0.0069	0.008
cis-16 18:1	0.071[b]	0.117[a]	0.085[b]	0.0096	0.021
∑ *cis* 18:1	7.15[c]	12.83[b]	15.48[a]	0.257	<0.001
∑ 20:1	0.011	0.017	0.016	0.0019	0.065
cis-13 20:1	0.016	0.024	0.024	0.0053	0.325
cis-15 24:1	0.053	0.060	0.057	0.0024	0.088
∑ MUFA[5]	15.6[b]	32.7[a]	32.1[a]	0.65	<0.001

[1] 50:50 F:C substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with *trans*-10 *trans*-14 18:2.
[5] MUFA = monounsaturated FA.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4.2.3. Polyunsaturated FA

The PUFA profile in the ruminal digesta of the 50:50 F:C substrate is presented in Table 14. Once again, without any intention of comparing substrates, the presence of 50% concentrate in this substrate raised *cis*-9 *cis*-12 18:2 content in the rumen digesta while decreasing *cis*-9 *cis*-12 *cis*-15 18:3. The former is the most abundant FA in cereal grains used

in ruminant diets, while the latter is more frequent in forages (Bauman et al., 1999; Glasser et al., 2013).

The supplementation with SBO resulted in a greater concentration of a number of UFA and PUFA (i.e., *cis*-9 *cis*-12 18:2, all individual CLA, *cis*-9 *cis*-12 *cis*-15 18:3, and total PUFA and UFA) compared to the other treatments, while higher percentages were obtained in TMO compared to PFA ($P < 0.05$). The differences observed in *cis*-9 *cis*-12 18:2 among the three treatments may arise from the variations in their FA profile. Concerning individual CLA and total CLA, the differences were similar to those discussed for the 100% F substrate. However, the lower *trans*-10 *cis*-12 CLA content in TMO compared to SBO supports the use of the former, *T. molitor* oil, as an alternative of the latter (SBO), as already mentioned by Renna et al. (2022a). Furthermore, the SBO treatment produced higher levels of *trans*-9 *cis*-11 and *trans*-10 *cis*-12 CLA, consistent with previous findings relating SBO and milk fat depression (Altenhofer et al., 2014; AlZahal et al., 2008). In terms of *cis*-9 *cis*-12 *cis*-15 18:3, the differences among treatments may arise from its percentages in the supplemental fats. Altogether, the results observed when the 50:50 was supplemented with 2% DM of TMO (decreased *trans*-10 18:1, and increased total *cis* 18:1, total MUFA, and total PUFA and UFA) would support the potential of using *T. molitor* oil to replace PFA and SBO to increase the energy density of dairy sheep diets and modulate the FA profile of ruminant meat and milk.

Table 14. Concentration (g/100 g FA) of polyunsaturated fatty acids of the ruminal digesta after 20 h incubation of the 50:50 F:C substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	50:50 F:C substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
cis-9 *cis*-12 18:2	3.52[c]	7.84[a]	5.62[b]	0.431	0.002
cis-11 *cis*-14 18:2	0.011	0.016	0.019	0.0049	0.423
trans-9 *trans*-12 18:2	0.046	0.055	0.043	0.0048	0.142
trans-9 *cis*-12 18:2	0.065[b]	0.128[a]	0.084[b]	0.0072	0.002
trans-11 *cis*-15 18:2[4]	0.200[c]	0.360[a]	0.229[b]	0.0046	<0.001
∑ non-conjugated 18:2	4.09[c]	8.70[a]	6.23[b]	0.418	0.001
cis-9 *trans*-11 CLA[5]	0.353[c]	0.722[a]	0.535[b]	0.0382	0.002
trans-9 *cis*-11 CLA	0.128[c]	0.278[a]	0.205[b]	0.0156	0.002
trans-10 *cis*-12 CLA	0.329[c]	0.519[a]	0.479[b]	0.0128	<0.001
trans-11 *trans*-13 CLA	0.046[c]	0.139[a]	0.065[b]	0.0029	<0.001
Other *trans trans* CLA	0.719[c]	1.835[a]	1.302[b]	0.0472	<0.001
∑ CLA	1.57[c]	3.49[a]	2.59[b]	0.096	<0.001
cis-9 *cis*-12 *cis*-15 18:3[6]	0.822[c]	1.239[a]	0.921[b]	0.0341	<0.001
trans-9 *trans*-12 *cis*-15 18:3	0.038	0.047	0.033	0.0040	0.054
∑ PUFA[7]	6.57[c]	13.53[a]	9.84[b]	0.453	<0.001
∑ UFA[8]	22.1[c]	46.2[a]	42.0[b]	1.06	<0.001

[1] 50:50 F:C substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with *trans*-10 *cis*-15 18:2.
[5] Contains *trans*-7 *cis*-9 and *trans*-8 *cis*-10 conjugated linoleic acid (CLA).
[6] Coelutes with *cis*-11 20:1.
[7] PUFA = polyunsaturated FA.
[8] UFA = unsaturated FA.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4.3. 10:90 F:C substrate

V.4.3.1. Saturated FA

The results of SFA in the ruminal digesta of 10:90 F:C substrate are reported in Table 15. Significant higher levels of 12:0 and 14:0 were found with TMO compared to the other treatments ($P < 0.05$). Both SFA, but particularly 14:0, have been shown to significantly increase low-density lipoprotein cholesterol concentrations in humans, thereby increasing

the risk of developing atherosclerosis (Calder, 2015; German & Dillard, 2004; Shingfield et al., 2008). On the other hand, PFA greatly increased the proportions of 16:0 and 18:0 when compared to SBO and TMO ($P < 0.05$), with the former being higher in TMO than in SBO ($P < 0.05$), and the latter being similar in both treatments ($P > 0.10$). These differences in even-chain FA proportions may arise from their presence in the supplemental lipids (e.g., 12:0 and 14:0 in TMO and 16:0 and 18:0 in PFA).

Regarding OCFA and BCFA, several FA were increased with TMO supplementation, such as 17:0 *anteiso* and 19:0, compared to the other treatments ($P < 0.05$), whereas 23:0 percentage was higher in TMO and SBO than in PFA ($P < 0.05$). Addition of TMO significantly increased total OCFA content compared to PFA ($P < 0.05$), with values statistically similar to those of SBO ($P > 0.10$). This increase in some OCFA and BCFA with *T. molitor* oil would suggest that this insect oil favoured some rumen bacteria producing these FA (Toral et al., 2020; Vlaeminck et al., 2006a). As a result, the ruminal accumulation of these FA could promote favourable changes in the meat and milk FA profile, which may be desirable from a human nutritional standpoint (Ran-Ressler et al., 2014; Shingfield et al., 2008). In addition, once again without intending to compare the substrates, it could be mentioned that increasing the concentrate proportion in the diet decreases the production of OCFA and BCFA in the rumen, most probably due to an increase in the proportions of starch-degrading bacteria and a reduction in cellulolytic bacteria (Bas et al., 2003; Vlaeminck et al., 2006a, b). The high FA content of the 10:90 F:C diet (28.3 vs. 17.1 and 15.4 mg FA/ g DM for the 10:90 F:C, 50:50 F:C and 100% F substrates, respectively) could also account for this decrease, as it has been reported that a high dietary FA content reduces *de novo* synthesis of these FA (Bionaz et al., 2020; Jenkins, 1993).

Table 15. Concentration (g/100 g FA) of saturated fatty acids of the ruminal digesta after 8 h incubation of the 10:90 F:C substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	10:90 F:C substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
12:0	0.050[b]	0.057[b]	0.143[a]	0.0116	0.002
13:0 *iso*	0.048	0.066	0.071	0.0121	0.257
13:0	0.036	0.025	0.047	0.0081	0.120
14:0 *iso*	0.036	0.042	0.066	0.0102	0.090
14:0[4]	1.188[b]	0.908[b]	2.312[a]	0.1078	<0.001
15:0 *iso*	0.076	0.089	0.110	0.0109	0.082
15:0 *anteiso*	0.338	0.363	0.436	0.0487	0.231
15:0	0.214	0.197	0.255	0.0163	0.052
16:0 *iso*	0.060	0.061	0.076	0.0078	0.175
16:0	44.3[a]	13.2[c]	15.4[b]	0.38	<0.001
17:0 *iso*	0.071	0.068	0.085	0.0071	0.138
17:0 *anteiso*	0.069[b]	0.077[b]	0.129[a]	0.0112	0.012
17:0	0.210	0.228	0.247	0.0138	0.136
18:0	12.6[a]	11.8[b]	11.2[b]	0.28	0.017
10-oxo-18:0[5]	0.268	0.233	0.243	0.0130	0.116
13-oxo-18:0	0.060	0.062	0.048	0.0113	0.463
19:0	0.042[b]	0.047[b]	0.074[a]	0.0056	0.009
20:0	0.379[b]	0.475[a]	0.398[b]	0.0073	<0.001
21:0	0.080[b]	0.094[a]	0.078[b]	0.0021	0.003
22:0[6]	0.302[c]	0.444[a]	0.370[b]	0.0059	<0.001
23:0[7]	0.114[b]	0.145[a]	0.147[a]	0.0066	0.013
24:0	0.206[b]	0.279[a]	0.253[ab]	0.0176	0.034
25:0	0.039	0.046	0.037	0.0038	0.126
26:0	0.148	0.156	0.159	0.0085	0.529
27:0	0.015[b]	0.022[a]	0.012[c]	0.0012	0.003
28:0	0.061	0.062	0.064	0.0054	0.911
∑ OCFA[8]	0.750[b]	0.804[ab]	0.896[a]	0.0384	0.046
∑ BCFA[9]	0.728	0.801	1.010	0.0953	0.085
∑ SFA[10]	60.8[a]	28.9[c]	32.2[b]	0.50	<0.001

[1] 10:90 F:C substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with DMA 15:0 *anteiso*.
[5] Coelutes with 9-oxo-18:0 as a minor component.
[6] Coelutes with a contaminant compound.
[7] Coelutes with geraniol.
[8] OCFA = odd-chain FA; calculated as the sum of fatty acids with an odd number of carbon atoms.
[9] BCFA = branched-chain FA; represents the summation of *iso* and *anteiso* fatty acids.
[10] SFA = saturated FA; all fatty acids with no double bonds.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4.3.2. Monounsaturated FA

Table 16 shows the monounsaturated FA profile of the ruminal digesta of the 10:90 F:C substrate. Both TMO and SBO showed a greater concentration of several *trans* 18:1 isomers (e.g., *trans*-5, *trans*-6+7+8, *trans*-9 18:1 and total *trans* 18:1) compared to PFA ($P < 0.05$). In addition, the TMO treatment led to similar *trans*-11 18:1 values than those of SBO ($P > 0.10$) and all these were higher than that of PFA ($P < 0.05$). The remarkable increase in *trans*-11 18:1 and total *trans* 18:1 isomers in SBO and *T. molitor* oil could be due to the high content of C18 PUFA in these oils. In addition, *trans*-10, *trans*-15 and *cis*-15 18:1 percentages did not differ among the three treatments ($P > 0.10$). Concerning *cis*-9 (+*trans*-13+14) 18:1 and total *cis* 18:1, their concentrations reached their greatest levels in TMO ($P < 0.05$), with SBO having significantly higher proportions than PFA ($P < 0.05$). As previously discussed for the supplementation of 100% F and 50:50 F:C substrates with TMO, the marked increase in *cis*-9 18:1, and total *cis* 18:1 and MUFA was related to the greater content of *cis*-9 18:1 in the *T. molitor* oil used in this study (about 39% of the total FA content). These results would also support the use of *T. molitor* oil in the diets of fattening lambs to modulate rumen BH and thus the FA profile of the meat and milk, especially since the higher levels of *trans*-11 18:1 was not accompanied by changes in *trans*-10 18:1 levels.

In general, increasing the concentrate proportion and PUFA levels in ruminant diets has been associated with a shift in the accumulation of different *trans* 18:1 in the rumen digesta (Ferlay et al., 2017; Griinari et al., 1998; Zened et al., 2012). Although this has been attributed to changes in the activity of *Butyrivibrio fibrisolvens* (Griinari & Bauman, 1999; McKain et al., 2010), this is nowadays known to be too simplistic (Toral et al., 2024). A shift towards the production of *trans*-10 18:1 happens in the rumen digesta of animals fed high starch diets supplemented with UFA-rich lipids (Zened et al., 2012, 2013), which is associated to low rumen pH, which favours alternative microbial populations that produce *trans*-10 18:1 isomer (Griinari & Bauman, 1999; Maia et al., 2007; Zened et al., 2012). In the current study, the inclusion of 90% concentrate in the diet resulted in relatively high proportions of *trans*-10 18:1 in the rumen digesta.

Table 16. Concentration (g/100 g FA) of monounsaturated fatty acids of the ruminal digesta after 8 h incubation of the 10:90 F:C substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	10:90 F:C substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
cis-7 16:1	0.090[b]	0.092[b]	0.460[a]	0.0112	<0.001
cis-9 16:1	0.089[b]	0.103[b]	0.577[a]	0.0065	<0.001
trans-4 18:1	0.111[c]	0.134[b]	0.152[a]	0.0032	<0.001
trans-5 18:1	0.114[b]	0.142[a]	0.151[a]	0.0040	0.002
trans-6+7+8 18:1	0.828[b]	1.07[a]	1.06[a]	0.048	0.012
trans-9 18:1	0.564[b]	0.635[a]	0.658[a]	0.0154	0.008
trans-10 18:1	1.96	2.17	2.11	0.125	0.349
trans-11 18:1	9.65[b]	11.86[a]	12.02[a]	0.318	0.003
trans-12 18:1	0.963[b]	1.131[a]	1.170[a]	0.0507	0.031
trans-15 18:1	0.610	0.918	1.291	0.1856	0.052
trans-16+*cis*-14 18:1[4]	0.191[b]	0.229[a]	0.236[a]	0.0103	0.023
trans-10/*trans*-11 18:1	0.206	0.185	0.177	0.0196	0.523
∑ *trans* 18:1	15.0[b]	18.3[a]	18.8[a]	0.63	0.007
cis-9+*trans*-13+14 18:1	12.2[c]	19.4[b]	25.5[a]	0.28	<0.001
cis-11 18:1	1.02[c]	1.74[a]	1.56[b]	0.017	<0.001
cis-12 18:1	0.470[b]	0.542[a]	0.576[a]	0.0151	0.005
cis-13 18:1	0.042	0.065	0.064	0.0072	0.053
cis-15 18:1	0.061	0.070	0.073	0.0055	0.201
cis-16 18:1	0.140[b]	0.152[ab]	0.164[a]	0.0057	0.034
∑ *cis* 18:1	13.9[c]	22.0[b]	27.9[a]	0.28	<0.001
∑ 20:1	0.018	0.025	0.029	0.0034	0.074
cis-13 20:1	0.018[b]	0.036[a]	0.018[b]	0.0037	0.014
cis-15 24:1	0.047	0.043	0.045	0.0046	0.725
∑ MUFA[5]	29.1[c]	40.6[b]	47.9[a]	0.63	<0.001

[1] 10:90 F:C substrate with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with *trans*-10 *trans*-14 18:2.
[5] MUFA = monounsaturated FA.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

V.4.3.3. Polyunsaturated FA

The results of the PUFA concentrations in the rumen digesta of the 10:90 F:C substrate can be found in Table 17. The percentage of *cis*-9 *cis*-12 18:2 was higher in TMO compared to PFA, although SBO showed the greatest percentage ($P < 0.05$). Once again, the marked difference in this FA among the three treatments would reflect their content in the

supplements. Regarding CLA, only *trans*-11 *trans*-13 CLA was affected, with a higher value in SBO than in TMO ($P < 0.05$), and PFA being similar to both SBO and TMO ($P > 0.10$). This greater concentration of *trans*-11 *trans*-13 CLA in the SBO treatment can reflect the high content of *cis*-9 *cis*-12 *cis*-15 18:3 in the SBO used in this study, which is the major source of *trans*-11 *trans*-13 CLA isomer (Ferlay et al., 2017; Toral et al., 2024). Moreover, proportions of *cis*-9 *cis*-12 *cis*-15 18:3 and total PUFA and UFA were found to increase from PFA, to *T. molitor* oil and SBO ($P < 0.05$). Based on the findings of this study, it seems that SBO and TMO inhibited several steps of the BH of dietary UFA when incorporated to the 10:90 F:C substrate. This is supported by: 1) the higher content in *cis*-9 *cis*-12 18:2 in SBO and *T. molitor* oil, 2) the lack of differences in individual and total CLA isomers (intermediates of *cis*-9 *cis*-12 18:2 and *cis*-9 *cis*-12 *cis*-15 18:3 rumen BH; Ferlay et al., 2017; Lee & Jenkins, 2011a, b), except for *trans*-11 *trans*-13 CLA, 3) the lack of variation in *cis*-15 and *trans*-15 18:1 isomers (intermediates of *cis*-9 *cis*-12 *cis*-15 18:3 BH; Ferlay et al., 2017; Jenkins et al., 2008), 4) the increase in total MUFA, and total PUFA proportions, and 5) the decline in 18:0 levels (the end product of rumen BH of C18 UFA; Griinari & Bauman, 1999; Harfoot & Hazlewood, 1997).

As already mentioned, the high proportion of concentrate in this 10:90 F:C substrate resulted in a high FA content, with *cis*-9 *cis*-12 18:2 representing about 44% of total FA. Therefore, this may have contributed to the observed high contents of this FA in the rumen digesta in this substrate (10:90 F:C).

Table 17. Concentration (g/100 g FA) of polyunsaturated fatty acids of the ruminal digesta after 8 h incubation of the 10:90 F:C substrate supplemented with the three fats (PFA, SBO and TMO)[1].

Item	10:90 FC substrate			SED[2]	*P*-value[3]
	+2% PFA	+2% SBO	+2% TMO		
cis-9 *cis*-12 18:2	6.49[c]	24.39[a]	15.78[b]	0.361	<0.001
cis-11 *cis*-14 18:2	0.014	0.014	0.012	0.0042	0.917
trans-9 *trans*-12 18:2	0.050	0.058	0.055	0.0048	0.353
trans-9 *cis*-12 18:2	0.099	0.123	0.120	0.0103	0.142
trans-11 *cis*-15 18:2[4]	0.194[b]	0.234[a]	0.216[a]	0.0067	0.011
∑ non-conjugated 18:2	7.08[c]	25.07[a]	16.49[b]	0.357	<0.001
cis-9 *trans*-11 CLA[5]	0.564	0.623	0.578	0.0837	0.777
trans-9 *cis*-11 CLA	0.097	0.093	0.081	0.0048	0.062
trans-10 *cis*-12 CLA	0.529	0.521	0.489	0.0717	0.848
trans-11 *trans*-13 CLA	0.059[ab]	0.066[a]	0.052[b]	0.0034	0.045
Other *trans trans* CLA	0.630	0.598	0.582	0.0285	0.333
∑ CLA	1.88	1.90	1.78	0.155	0.740
cis-9 *cis*-12 *cis*-15 18:3[6]	0.649[c]	2.958[a]	1.077[b]	0.0570	<0.001
trans-9 *trans*-12 *cis*-15 18:3	0.023	0.032	0.026	0.0032	0.123
∑ PUFA[7]	9.65[c]	29.98[a]	19.44[b]	0.354	<0.001
∑ UFA[8]	38.8[c]	70.6[a]	67.3[b]	0.50	<0.001

[1] 10:90 F:C substrate supplemented with either 2% DM of palm distillate fatty acids (PFA), soybean oil (SBO) or *Tenebrio molitor* oil (TMO).
[2] SED = standard error of the difference.
[3] Probability of significant effects of the experimental treatment.
[4] Coelutes with *trans*-10 *cis*-15 18:2.
[5]Contains *trans*-7 *cis*-9 and *trans*-8 *cis*-10 conjugated linoleic acid (CLA).
[6] Coelutes with *cis*-11 20:1.
[7] PUFA = polyunsaturated FA.
[8] UFA = unsaturated FA.
[a-c] Within a row, different letters indicate statistical differences among the groups ($P < 0.05$).

Final remarks

Overall, the results of this study support that *Tenebrio molitor* oil, at 2% of diet DM, could replace other lipid sources (i.e., those derived from palm and soybean) that are associated with a high environmental impact and are subject to market fluctuations.

This *in vitro* study, analysing the effect of *T. molitor* oil on rumen fermentation and biohydrogenation when compared to the same levels of palm distillate fatty acids and soybean oil, represents a first step toward incorporating this alternative lipid source into ruminant diets.

However, several aspects remain to be explored, such as the *in vivo* effects, including those on animal performance and product quality, and with particular attention to the fatty acid composition of meat and milk (considering the overall lipid profile, not just the specific impact on individual fatty acids). Further research will also be recommended to determine, for example, the optimal inclusion level and the interactions between the insect fat and the composition of the basal diet. Additionally, it would be important to investigate whether these first results can be extrapolated to other production contexts, and to conduct a more holistic evaluation of the use of insect products in livestock across different production and economic frameworks. In any event, these are just proposals for future research and lie beyond the scope of this MSc thesis.

VI. CONCLUSIONS

First. The inclusion of *Tenebrio molitor* oil at 2% diet DM hardly causes any effects on *in vitro* ruminal fermentation and degradation across the three diets studied (i.e., diets with forage-to-concentrate ratios of 100:0, 50:50, and 10:90). If any effects are observed, when comparing the inclusion of the insect oil with that of soybean oil or palm distillate fatty acids, they are minor. These results suggest that *T. molitor* oil could be considered a potential alternative lipid source in ruminant feeding.

Second. The effects of *T. molitor* supplementation on ruminal biohydrogenation show a little more variation depending on the basal diet. However, several consistent patterns are also identified across the 3 diets studied.

Third. The higher proportions of odd-chain fatty acids (OCFA) in the rumen digesta of substrates supplemented with *T. molitor* oil (TMO), compared to those supplemented with palm distillate fatty acids (PFA), suggest that this treatment fosters a more favourable ruminal environment for the microbiota involved in the synthesis of these bioactive lipids, which are associated with potential health benefits in humans. Results are similar to those observed with soybean oil supplementation (SBO).

Fourth. Most of the effects on the fatty acid profile of the rumen digesta induced by supplementation with *T. molitor* oil can be attributed to the specific fatty acid composition of this lipid source, as occurs with PFA and SBO supplementation. These effects seem to happen: **i)** directly, through the transfer of the major fatty acids from the lipid supplements to the rumen digesta (e.g., 16:0 with PFA, *cis*-9 18:1 and 14:0 with TMO, and *cis*-9 *cis*-12 18:2 with SBO), or **ii)** indirectly, via the biohydrogenation of unsaturated FA contributed by the supplements (e.g., higher concentrations of some intermediates, such as certain conjugated linoleic acid -CLA- isomers or *trans*-MUFA, with SBO and TMO, and of 10-oxo-18:0 with TMO).

Fifth. Supplementation with *T. molitor* oil increases *trans*-11 18:1 concentration compared to palm distillate fatty acids. However, it also leads to higher levels of *trans*-10 18:1 in the 100% F and 50:50 F:C substrates. Notably, no shift in the *trans*-10/*trans*-11 ratio is observed in any case. Similar patterns are found for *cis*-9 *trans*-11 CLA in the same

substrates (100% F and 50:50). These results are similar or slightly less favourable than those obtained when the diet is supplemented with SBO. However, in some cases, SBO may cause higher increases not only in *trans*-10 18:1 but also in *trans*-10 *cis*-12 CLA, both potentially detrimental for dairy ruminants.

VII. REFERENCES

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