UNIVERSIDAD DE LA REPÚBLICA

FACULTAD DE VETERINARIA

Programa de Posgrados

MANEJO DIFERENCIAL EN EL POSTPARTO TEMPRANO DE VACAS LECHERAS: IMPACTO SOBRE LA PRODUCCIÓN, PARÁMETROS ENDÓCRINO-METABÓLICOS Y REPRODUCTIVOS

GRACIANA RODRIGUES MENDINA

TESIS DE DOCTORADO EN PRODUCCIÓN ANIMAL

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GRACIANA RODRIGUES MENDINA, DCV, MSc.

Ana Meikle, DMTV, MSc., PhD.

Directora de Tesis

María de Lourdes Adrien, DCV, MSc., PhD.

Victoria de Brun, Lic., MSc., PhD

Eugenio Jara, Lic., MSc., PhD

Co-Directores de Tesis

2025

INTEGRACIÓN DEL TRIBUNAL DE DEFENSA DE TESIS

Alejandro Mendoza (PhD)
Programa de Producción de Leche
Instituto Nacional de Investigación Agropecuaria
Uruguay

Mariana Carriquiry (PhD)
Departamento de Producción Animal y Pasturas,
Facultad de Agronomía, Udelar
Uruguay

Maria Belén Rabaglino (PhD)
Farm Animal Health, Department Population Health Sciences
Utrecht University
The Netherlands

2025

ACTA DE DEFENSA DE TESIS DE DOCTORADO

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Tribunal:

Presidente: PhD Ing. Agr. Alejandro Mendoza

2° Integrante: Dra. (PhD) Belén Rabaglino

3er Integrante: PhD Ing. Agr. Mariana Carriquiry

C.I.	Nombre	Concepto	Nota
5.873.183 - 9	Graciana, Rodrigues Mendina	Excelente	E

Nota: La calificación mínima para aprobar la defensa es Aceptable (A)

La tesis de la DCV MSc Graciana Mendina aborda una problemática relevante y poco estudiada para las condiciones del país, buscando entender la respuesta biológica a distintos manejos de alimentación y de infraestructura en vacas lecheras La tesis comprende la realización de 2 experimentos complejos, y cuenta con 2 artículos ya publicados como primera autora. El material escrito que fue entregado es de muy alta calidad, integrando de forma fluida la información generada y contrastándola con la disponible en la literatura nacional e internacional. La presentación oral también fue muy clara y sintética. En el intercambio con el tribunal la estudiante demostró que conoce los resultados obtenidos en profundidad, mostrando solvencia al momento de contestar las distintas preguntas que le fueron formuladas. Por todo lo anterior, el tribunal de forma unánime le concede la calificación de EXCELENTE.

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Ing Agr PhD Alejandro Mendoza	**ALEJANDRO FRANCISCO MENDOZA AGUIAR** PAÍS: URUGUAY FECHA: 2025.08.22 | 12:29:27 -03:00 **FIRMA ELECTRÓNICA AVANZADA | VALIDEZ LEGAL: LEY 18.600** FIRMA RESPALDADA POR IDfirma Abitab
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“Look deep into nature, and then you will understand everything better.”

Albert Einstein

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Aunque el camino del doctorado es por momentos solitario, estando largas horas sentado en frente a una computadora mientras tu gente disfruta del ocio envidiable, y te enfrentas a los desafíos de superación que solo dependen de ti, todo eso es posible gracias a otros. Otros que estuvieron antes, o que lo están transitando a tu lado. No puedo empezar de otra manera que no sea agradeciendo a mi orientadora en este proceso, Ana. Si terminé ingresando a la academia, fue porque tu plantaste esa semilla aquel 21 de diciembre de 2017, cuando integraste mi tribunal de defensa de tesis de grado. La conexión que sentí al escuchar mis pensamientos saliendo por otra boca me hizo saber que, si algún día volvía, quería trabajar contigo. Capaz hay cosas que están destinadas a ser, ya sea por los giros inesperados que después cobran sentido, o cuando todo fluye de manera fácil. Y si bien entre nosotras fue relativamente fácil, porque nos entendemos y nos aceptamos, no fue menos desafiante. Con interpelaciones frontales y efervescentes, las primeras veces avasalladoras, pero en seguida aprendí tu lenguaje – y a traducir tus terribles abreviaturas y errores de tipeo- y junto con Mateo fuimos entendiendo cómo llevar las riendas (y también a mascar el freno cuando tocaba). Sí, es imposible no incluir a Mateo en esta relación, con quien hicimos este camino en paralelo con subidas y bajadas (y buenos golpes en esas últimas). Entre los tres construimos ese espacio profundo y *muy fuerte* desde aquel ICAR en Bologna, que terminó superando la relación tutor-estudiantes. Buscaste transmitirnos una sólida formación académica desde un lugar donde tendemos a sentir que no somos tan buenos como en las más renombradas universidades (la humilde idiosincrasia uruguaya). Utilizaste todos tus recursos para permitirnos alzar vuelo e intercambiar a la par con científicos de excelencia. Pero además, estuviste atenta a nuestra formación personal, compartiendo tus propias vivencias y fragilidades en el interín. Nos permitiste ver más allá de la cáscara intimidante que muchos perciben, y conocer a la Ana real que aún se preocupa por mejorarse, que en el acierto o en el error trata de ser fiel a lo que siente ser mejor para el todo, siempre con pasión y convicción. El impacto que has tenido en mi vida, y en la de muchos otros alrededor, muchas veces de manera anónima, es algo que seguramente todavía no puedo dimensionar. A la par está Lourdes, que me viene acompañando desde la tesis de grado y por lo tanto ya no queda tanto por decir, a no ser agradecer por seguir al pie del cañón haciendo el dos para lo que sea necesario, así sea trabajar en horas y condiciones ambientales poco amigables, ser oído comprensivo para un desahogo, y nunca permitir que me falte nada para seguir creciendo y cumpliendo metas… qué suerte es poder trabajar a tu lado! Gracias también a Vicky de Brun por guiarme en el laboratorio de biología molecular, y a Eugenio por darme las primeras nociones de bioinformática. También debo volver a agradecer a mi co-tutor de maestría, Juan Pablo, quien participó de la construcción del primer capítulo de esta tesis y contribuyó de manera generosa a mi formación científica y manejo de la estadística. En este sentido, otro gran agradecimiento a Coco Bentancur, quien siempre nos ha dado soporte para los análisis estadísticos y manejo de SAS.

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PROPOSICIONES

I. La vaca lechera Holando multípara posee una gran capacidad adaptativa al ambiente productivo, logrando transformar el incremento en cantidad y densidad de nutrientes en más leche, en lugar de destinarlo a mejorar el estatus energético en los primeros días postparto, o en adelantar el reinicio de su ciclicidad (de esta Tesis).

II. La vaca primípara que llega a su primer parto e ingresa al tambo con 2 años de edad -aunque igualmente generosa- necesita culminar su crecimiento, pero el estatus anabólico puede verse perjudicado en un sistema de base pastoril respecto a un estabulado con TMR, al inicio de la lactancia (de esta Tesis).

III. La vaca lechera es de las mejores maestras en producción animal, siendo el estudio de su función de lujo, la reproducción, una variable sensible para medir nuestras decisiones de manejo, aunque pasible de ser recubierta por su gran capacidad de resiliencia; cualidad también encontrada en sus productores.

IV. Una vez me enseñaron que el secreto de los tambos de éxito se basa en: **A**limentación, **M**anejo, **O**rganización, **R**eproducción (Luís Mário Mendina, de los tamberos más apasionados que he conocido).

V. Es necesario estar absolutamente presente para ganar un punto en tenis/pádel: quedar pensando en el punto anterior perdido, ganado, o en el que viene, lleva al error; ese punto es lo más importante en ese momento y no depende tanto de lo que haga tu oponente, si no de como llegas a la pelota, y aún siendo el número 1 del mundo, vas a acertar solamente el 54% de los puntos, pudiendo aun ser victorioso en el 80% de los partidos… y esto no es solo sobre tenis.

VI. La comunicación, con todos sus recursos, incluido el silencio, es un arma poderosa: el lenguaje cambia y adopta nuevas palabras a lo largo del tiempo y espacio, por eso, independiente de la lengua materna (ya bastante oxidada), la de alfabetización (que recibe palabras que yo creo que existen) o si arañas un pobre inglés, aun así, es posible conectar con otra alma… al fin de cuentas: “Every act of communication is an act of translation” (Gregory Rabassa).

ESTRUCTURA DE LA TESIS Y PUBLICACIONES

La presente tesis está basada en tres capítulos, correspondientes a tres artículos científicos que se detallan a continuación:

I. *Metabolic adaptation to lactation of dairy cows in two contrasting facilities involving partial confinement plus grazing or total confinement.* **Mendina GR**, Damián P, Meikle A, Mendez, MN, Chilibroste P, Adrien L. Animal Production Science 64 (2024) AN23383. doi: 10.1071/AN23383.

II. *Feeding strategies during fresh cow period in pasture-based dairy systems: metabolic adaptation to lactation and resumption of ovarian cyclicity in primiparous and multiparous cows.* **Mendina GR***,* Adrien ML, López-Radcenco A, Moyna G, Rivoir C, Chilibroste P, Meikle A. En revisión en Journal of Dairy Science.

III. *Is there any residual effect of differential feeding strategies during the fresh period on endometrium transcriptomics and metabolomics at the end of the voluntary waiting period in the dairy cow?* **Mendina et al.** Manuscrito.

Durante la formación doctoral, se ha realizado otro experimento que reporta el uso de un biomarcador para el diagnóstico de preñez temprana en vaquillonas lecheras que se anexa al final de esta tesis: *Increased expression of interferon-stimulated gene 15 (ISG15) in cervical cells on day 14 of pregnancy in Holstein heifers.* **Mendina GR**, de Brun V, Adrien ML, Pons V, Vivian Paradizo R, Gil J, Rocha CC, Binelli M, Meikle A. (2025) JDS Communications. 6:165–170. doi: 10.3168/jdsc.2024-0596.

Otros trabajos asociados a esta tesis son:

Performance of autumn- and spring-calving Holstein dairy cows confined indoors or managed with pasture and supplementation under various housing conditions. Menegazzi G, **Mendina GR**, Grille L, Méndez MN, Pons V, Pedemonte A, Adrien ML, Meikle A, Gerrits WJJ, Dijkstra J, Chilibroste P. (2025) Journal of Dairy Science. Apr 28:S0022-0302(25)00275-9. doi: 10.3168/jds.2024-26084.

Contrasting feeding management in the first 21 days pospartum in Holstein dairy cows: direct and residual milk responses. Rivoir C, **Mendina G**, Adrien ML, Meikle A, Chilibroste P. Animal Open Space (2025). doi: 10.1016/j.anopes.2025.100092.

TABLA DE CONTENIDO

RESUMEN

Esta tesis tuvo como objetivo general evaluar el impacto de las condiciones de infraestructura durante el encierro parcial y estrategias de alimentación durante la lactancia temprana, sobre la adaptación endócrino-metabólica postparto, y sus consecuencias sobre la performance productiva, el reinicio de ciclicidad y el ambiente uterino al momento de iniciar los servicios de vacas lecheras en sistemas pastoriles intensivos. El trabajo se estructuró en tres capítulos, correspondientes a tres artículos científicos. En el experimento I (artículo I), se evaluó el efecto de distintas condiciones de infraestructura (cielo abierto vs. galpón de *compost*) durante el encierro parcial utilizado en combinación con pastoreo, a su vez comparados con un sistema de estabulación completa en galpón de *compost* con dieta mezcla total (TMR) *ad libitum*. Ambos sistemas basados en pastoreo (cielo abierto y galpón de *compost*) presentaron menor nivel de producción aunque mayor proporción de vacas ciclando en el postparto temprano respecto al grupo estabulado con TMR *ad libitum*. Las vacas con encierro parcial a cielo abierto presentaron mayores concentraciones de β-hidroxibutirato a los 60 y 90 días en leche (DIM), indicativos de cetosis subclínica, comparado a los otros dos grupos, lo cual pudo deberse al mayor nivel de exposición ambiental asociado a estrés calórico. En el experimento II (artículos II y III), se evaluó el efecto de un manejo alimenticio diferencial estratégico durante los primeros 21 DIM basado en estabulación con TMR *ad libitum* comparado a pastoreo con suplementación con PMR desde el parto en vacas primíparas y multíparas. Luego del tratamiento diferencial, ambos grupos se manejaron en pastoreo con suplementación. Las vacas multíparas respondieron incrementando la producción de leche ante una mayor ingesta de materia seca y energía, manteniendo similares niveles de movilización de reservas corporales respecto a vacas en pastoreo. Por otro lado, este manejo diferencial no se asoció con mayor producción de leche en la vaca primípara, pero sí en un mejor estado energético (perfiles metabólicos y endócrinos) respecto a las vacas en pastoreo, cuya endocrinología metabólica reflejó mayor dificultad en la adaptación a la lactancia bajo condiciones pastoriles. Por otra parte, el cambio de manejo abrupto al pastoreo luego del periodo de encierro generó una readaptación metabólica en ambas paridades, que se apoyaron en el uso de sus reservas corporales y ajustaron a la baja la salida energética en leche. La evidencia obtenida a partir del experimento I apuntaría a que las vacas multíparas priorizan el direccionamiento de la energía extra de la TMR hacia producción de leche, relegando temporariamente el reinicio de la actividad ovárica, mientras que la aplicación de estabulación con TMR durante 21 DIM, no fue suficiente para generar diferencias en términos de probabilidad de reinicio respecto al grupo en pastoreo. El efecto residual de este manejo estratégico al momento de iniciar los servicios sobre el endometrio es limitado, una vez transcurridos 40 días de sufrir un manejo similar bajo pastoreo con suplementación. Sin embargo, algunas diferencias en indicadores metabolómicos y transcriptómicos podrían asociarse al impacto metabólico ocasionado por los manejos alimenticios o el cambio de la estabulación al pastoreo. Los resultados de esta tesis resaltan la importancia del manejo y el ambiente productivo durante el posparto temprano, sobre la adaptación endócrino-metabólica a la lactancia y la respuesta productiva en vacas lecheras primíparas y multíparas.

SUMMARY

The general objective of this thesis was to evaluate the impact of housing conditions during partial confinement and feeding strategies during early lactation on the endocrine-metabolic adaptation to lactation, and their consequences on productive performance, resumption of ovarian cyclicity, and the uterine milieu at the beginning of the breeding period in dairy cows under intensive pasture-based systems. The work was structured into three chapters, corresponding to three scientific articles. In Experiment I (Article I), the effect of different housing conditions (outdoor soil-bedded vs compost barn) during partial confinement in combination with grazing was evaluated, and both were compared with total confinement in a compost barn with *ad libitum* total mixed ration (TMR). Both pasture-based systems (outdoor soil-bedded and compost barn) showed lower milk production levels but a higher proportion of cycling cows during early postpartum compared to the total confinement TMR system. Cows in outdoor partial confinement showed higher concentrations of β-hydroxybutyrate at 60 and 90 days in milk (DIM), indicative of subclinical ketosis, compared to the other two groups, possibly due to greater environmental exposure and heat stress. In Experiment II (Articles II and III), the effect of a strategic feeding management during the first 21 DIM was evaluated, comparing confinement with *ad libitum* TMR versus grazing with partial mixed ration (PMR) supplementation from calving, in both primiparous and multiparous cows. After the differential treatment period, both groups were managed on pasture with supplementation. Multiparous cows responded by increasing milk yield in response to greater dry matter and energy intake, while maintaining similar levels of body reserve mobilization compared to grazing cows. Conversely, this differential management was not associated with higher milk production in primiparous cows. Still, it did result in better energy status (as reflected by metabolic and endocrine profiles) compared to grazing cows, whose metabolic endocrinology suggested greater difficulty adapting to lactation under grazing conditions. Moreover, the abrupt change in management from confinement to grazing induced a metabolic readjustment in both parities, relying on body reserves and downregulating milk energy output. Evidence from Experiment I suggests that multiparous cows prioritize directing the extra energy from TMR toward milk production, temporarily delaying the resumption of ovarian cyclicity. Meanwhile, confinement with TMR for 21 DIM was not sufficient to generate differences in the probability of resumption of ovarian cyclicity compared to the grazing group. The residual effect of this strategic management on the endometrium at the beginning of the breeding period appears to be limited after 40 days under similar grazing and supplementation conditions. However, some differences in metabolomic and transcriptomic indicators may be associated with the metabolic impact of the feeding strategies or the transition from confinement to grazing. The results of this thesis highlight the importance of postpartum management and production environment on endocrine-metabolic adaptation to lactation and the productive response of primiparous and multiparous dairy cows.

1. INTRODUCCIÓN

Frente a la creciente demanda de alimentos, los sistemas de producción lechera se han intensificado a nivel mundial (Fariña & Chilibroste, 2019; Hennessy et al., 2020; Ma et al., 2020; Moscovici Joubran et al., 2021). El sector lechero uruguayo ha seguido similar tendencia, y pese a un contexto de precios volátiles y competencia por tierra, traducido en una disminución de remitentes y del área destinada a la lechería, se ha incrementado la productividad (Fariña & Chilibroste, 2019; Chilibroste et al., 2024). La mejora en dicha productividad se ha basado en el aumento de la carga animal y de la producción individual, apoyado por mejoras genéticas, de manejo y alimentación, con el uso creciente de suplementación (Fariña & Chilibroste, 2019; Chilibroste et al., 2024).

La mayor carga animal permite aumentar la utilización de las pasturas reduciendo los costos de alimentación (Baudracco et al., 2010). Sin embargo, esto también repercute en un aumento del tiempo en que los animales no pueden acceder a los potreros para el pastoreo directo, ya sea por el bajo stock de forraje o por condiciones de piso (Ortega et al., 2024). Esto lleva a que los animales deban permanecer en otros lugares y recibir suplementación para completar sus requerimientos nutricionales (Baudracco et al., 2011; Ortega et al., 2024). Los lugares donde se alojan los animales mientras no están pastoreando poseen condiciones diversas en el país (Aguerre et al., 2018), pero son en su mayoría a cielo abierto (Chilibroste & Battegazzore, 2019). La exposición a las condiciones ambientales (estrés calórico, lluvias, formación de barro, etc.) pueden desencadenar mecanismos adaptativos en el metabolismo de los animales (Kadzere et al., 2002; De Rensis & Scaramuzzi, 2003; Tucker et al., 2007; Das et al., 2016) y amenazar su bienestar (Pereira et al., 2017; Mendina et al., 2023; Pons et al., 2023). Los sistemas de base pastoril intensivos están sujetos a cambios de manejo que impactan al animal de manera diferencial dependiendo de la etapa del ciclo productivo. Si estos estresores se dan en momentos especialmente vulnerables para la vaca lechera como durante la adaptación metabólica a la lactancia temprana, esto podría perjudicar aún más su performance productiva y reproductiva.

El periodo de transición, es decir, el pasaje de un estado preñado no lactante a uno no preñado en lactancia ha sido arbitrariamente definido como el tiempo que ocurre entre las tres semanas previas y posteriores al parto (Grummer et al., 2004). Este período es considerado como el más desafiante en la vida de la vaca lechera. La alta demanda energética para producción de leche que no es cubierta con su limitada capacidad de consumo alrededor del parto, resulta en un balance energético negativo (BEN) fisiológico (Drackley, 1999). Dado que la magnitud del BEN ha sido asociada negativamente con la salud, producción y reproducción (Patton et al., 2007; Macmillan et al., 2020; Stevenson et al., 2020), se han explorado diversas estrategias nutricionales para intentar atenuarlo (Cardoso et al., 2020). Las evidencias parecen indicar que animales en sistemas pastoriles presentan un BEN más pronunciado que animales en sistemas estabulados comiendo dieta mezcla total (TMR; Kolver & Muller, 1998; Bargo et al., 2002; Meikle et al., 2013). Sin embargo, la literatura que evalúa los actuales sistemas que combinan pastoreo y suplementación con dieta mezcla parcial (PMR) es escasa (Bargo et al., 2002; Mendoza et al., 2016a; Salado et al., 2018, 2020) y pocos se han enfocado en el periodo más crítico de lactancia temprana (Astessiano et al., 2015; Fajardo et al., 2015). Aún más, estrategias de estabulación de corta duración focalizadas en la etapa postparto del periodo de transición (primeros 21 días postparto) han sido menos exploradas (Al Ibrahim et al., 2013; Brady et al., 2021). Estos antecedentes indicarían beneficios para vacas estabuladas en los primeros días postparto (primeros 21 o 30 días postparto) en cuanto a indicadores metabólicos, pero sin diferencias en producción de leche (Al Ibrahim et al., 2013; Brady et al., 2021). En cuanto a parámetros reproductivos, no se detectaron diferencias en cuanto a días a la primera ovulación bajo estas estrategias de estabulación de corta duración respecto a vacas en pastoreo desde el parto, que rondó alrededor de 20 días en ambos tratamientos (Brady et al., 2021), o tendió a ser inferior para vacas estabuladas (19 vs 24 días; Al Ibrahim et al., 2013). No obstante, no se evaluó el impacto de este manejo para las distintas paridades (primíparas vs multíparas), teniendo en cuenta que el potencial de partición de nutrientes está asociado a la categoría animal (Wathes et al., 2006). Además del desafío metabólico, las vacas primíparas también se enfrentan al estrés novedoso del parto y rutina de ordeñe (Eicher et al., 2007), que asociado al nuevo manejo alimenticio expuesto a la competencia con las vacas multíparas, podría perjudicar su adaptación y performance.

Usualmente las vacas lecheras bien manejadas presentan su primera ovulación dentro del primer mes posparto (Crowe et al., 2014), sin embargo, las tasas de concepción son bajas a nivel internacional (Rhodes et al., 2003; Santos et al., 2009) y a nivel nacional (Cruz et al., 2024). La mayoría de las pérdidas de preñez (29%) en sistemas pastoriles ocurre en la primer semana de gestación (Berg et al., 2022). En sistemas confinados se ha reportado similar distribución (10-50%) y se ha atribuido como una de las principales causas los problemas metabólicos postparto (Wiltbank et al., 2016). En estas etapas previas a la implantación, el desarrollo del embrión depende del histotrofo, definido por el conjunto de moléculas secretadas por el endometrio o transportadas al lumen que actúan como combustible y cofactores de diversos procesos (Roberts & Bazer, 1988; Binelli et al., 2022). Se ha sugerido que el endometrio podría censar el estatus metabólico del animal adaptando su fisiología (Meikle et al., 2018). Sin embargo, la comprensión del funcionamiento uterino al momento del servicio en vacas lecheras al inicio de lactancia y su vínculo con el estatus endócrino-metabólico aún es limitada. Vacas lactantes con distinto grado de balance energético negativo, presentaron expresión diferencial de genes en el tracto reproductivo durante el posparto temprano (Wathes et al., 2006) y al momento de realizar los servicios (Valour et al., 2013), vinculados a remodelación tisular y respuesta inmune, funciones ligadas al mérito genético para fertilidad (Moran et al., 2017). Antecedentes nacionales en vacas primíparas sugieren una expresión génica endometrial más favorable para el éxito de la preñez en animales con mejor nivel nutricional (Astessiano et al., 2017). Además, existen evidencias de que el metaboloma uterino se modifica ya en los primeros días del ciclo o gestación, adaptándose a las necesidades del embrión (Sponchiado et al., 2019; Tríbulo et al., 2019). Entre los metabolitos asociados a diferencias en la fertilidad o éxito en la preñez, una gran variedad podría ser afectado por el tipo de alimentación recibida por los animales o por su estatus metabólico (Fair, 2014). Sin embargo, para nuestro conocimiento no existen estudios que hayan evaluado si el manejo alimenticio durante los primeros 21 días postparto podría afectar de manera residual tanto el transcriptoma como el metaboloma uterino al momento de iniciar los servicios.

2. ANTECEDENTES ESPECÍFICOS

2.1. Sistemas de producción lechera en el mundo y en Uruguay

Los sistemas de producción ganadera se han intensificado a nivel mundial, estimulados por la creciente demanda de alimentos (Fariña & Chilibroste, 2019; Hennessy et al., 2020; Ma et al., 2020; Moscovici Joubran et al., 2021). Los países con una lechería más intensificada fueron responsables del 45,4% de la leche producida a nivel mundial, utilizando el 15,4% de las vacas lecheras en el mundo, en el año 2018 (Britt et al., 2021). Con algunas vacas produciendo más de 14 mil kg de leche por año, y picos de producción de más de 60 kg/día, el ajuste preciso de la alimentación se vuelve esencial (Britt et al., 2021). Se estima que actualmente entre el 85 y 90% de la leche producida a nivel mundial proviene de sistemas confinados (Shalloo et al., 2018). Sin embargo, existe un resurgimiento del interés en los sistemas basados en pastura, impulsado por las tendencias del mercado y las preferencias de los consumidores (Jackson et al., 2020; Moscovici Joubran et al., 2021). Además de considerar el pastoreo de las vacas como un atributo de bienestar (Jackson et al., 2020), existe evidencia sólida de que los animales alimentados a pasto producen alimentos con componentes nutracéuticos beneficiosos para la salud humana (O'Callaghan et al., 2016; Barca et al., 2017; Stanton et al., 2021; Timlin et al., 2023). Por lo tanto, explorar el sistema natural de alimentación de los bovinos en base a pastoreo, también presenta cualidades en cuanto a posicionamiento de mercado.

El sector lechero uruguayo destina más del 70% de su producción al mercado internacional, contribuyendo significativamente con la economía nacional (DIEA, 2024). Entre los países con producción de base pastoril, Uruguay es el segundo con mayor crecimiento en la producción de leche en los últimos 30 años (1991-2021), posicionándose luego de Nueva Zelanda (Chilibroste et al., 2024). Cambios dramáticos han marcado ese lapso, con una disminución en el número de productores de 49% (de 6516 a 3320), y en el área de 29% (de 1.064.000 a 757.000 ha). No obstante, la productividad presentó un aumento vertiginoso de 223%, pasando de 82 a 341 kgMS/ha/año de sólidos (grasa + proteína) en las últimas 3 décadas (Chilibroste et al., 2024). Este aumento de la productividad es explicado en gran medida por la mejora en la producción individual y el aumento en la carga animal (Fariña & Chilibroste, 2019), resultado del aumento del 15% en el número de vacas en producción (de 357.000 a 421.000), al mismo tiempo en que el área destinada a la lechería se redujo (Chilibroste et al., 2024).

La carga animal promedio de los sistemas relevados por un proyecto CONAPROLE, fue de 1,15 VM/ha, pero con amplia variabilidad entre sistemas, de 0,5 a 2,0 VM/ha (Chilibroste & Battegazzore, 2019). Además del impacto en productividad, tener más animales por ha, permite una mayor cosecha de pasto (Baudracco et al., 2011), abaratando el costo de alimentación y mejorando la rentabilidad de los sistemas (Fariña & Chilibroste, 2019; Chilibroste et al., 2024). Pero el aumento de la carga animal también aumenta la necesidad de suplementación para cubrir los requerimientos nutricionales individuales (Baudracco et al., 2010; Vibart et al., 2012). En Uruguay, un estudio de *farmlet* de 3 años, demostró que a medida que la carga animal aumenta de 1,5 a 2,0 VO/ha, aumenta el uso de suplementación y disminuye la frecuencia en que los animales logran salir a pastorear en dos o incluso una vez al día, ya sea por condiciones de piso o limitado stock de forraje (Ortega et al., 2024).

Estos cambios marcados en la alimentación, así como en la selección genética animal y mejoras de manejo, han permitido no solo el aumento de la productividad, sino también de la producción individual. La vaca lechera en Uruguay, pasó de estar en un sistema netamente extensivo, produciendo 11,5 L/día en el año 1993, a producir 19,3 L/día en promedio en la lactancia en 2023 (DIEA - OPYPA, 1999; DIEA, 2024), con un sistema de alimentación y ambiente marcado por la intensificación. No obstante, los sistemas de mayor productividad parecerían estar alcanzando techos de crecimiento, reflejándose en el estancamiento de la producción anual en la última década (Chilibroste et al., 2024).

En un contexto de competencia por tierra, en donde el 66% del área explotada para lechería no es propia (DIEA, 2023), el aumento de la carga animal parece alcanzar ciertos límites o inducir a sistemas confinados. En este proceso, y siguiendo la tendencia mundial, un pequeño grupo de productores ha optado en los últimos años por sistemas de confinamiento con TMR, buscando una mayor productividad por hectárea y estabilidad a lo largo del año. Sin embargo, a pesar del potencial de incremento en producción, los costos también se incrementan, siendo igualmente desafiante lograr márgenes económicos sostenibles (Menegazzi et al., 2025). Para mantenerse en la actividad, sea cual fuere el sistema elegido, los productores se ven obligados a mejorar la eficiencia para mantener su competitividad. Ultrapasar las marcas actuales de producción individual implica afinar procesos, mirando a las necesidades fisiológicas de la vaca en términos nutricionales, de salud, y confort ambiental, para alcanzar el mejor desempeño en su ciclo productivo.

2.2. Sistemas de alimentación y encierros

Gran parte de los sistemas uruguayos netamente pastoriles fueron migrando hacia sistemas mixtos que combinan el pastoreo directo con suplementos. El uso de suplementación permite cubrir de mejor manera los requerimientos nutricionales de la genética actual, que estarían incompletos con pastoreo exclusivo, alcanzando mayores niveles de producción (Kolver & Muller, 1998). Se ha relevado que la dieta de las vacas en los establecimientos lecheros uruguayos está compuesta de un 25% de reservas y 18% de concentrados, mientras el pastoreo directo representa el 57% de la dieta (Chilibroste & Battegazzore, 2019). El concentrado es suministrado en la sala de ordeñe en el 85% de las matrículas, mientras que la reserva de forraje se suministra en comederos a cielo abierto (Chilibroste & Battegazzore, 2019). Proporcionar esos suplementos mezclados como PMR es otra opción posible y utilizada (Fajardo et al., 2015; Mendoza et al., 2016). A medida que aumenta la carga animal, y por lo tanto el uso de suplementación y tiempo de permanencia de los animales en los lugares de encierro (Ortega et al., 2024), nuevos desafíos en las condiciones productivas empiezan a surgir. Las condiciones de infraestructura de esos lugares son diversas (Aguerre et al., 2018) y representan un punto de preocupación en cuanto al impacto sobre bienestar animal y consecuente repercusión en la eficiencia de los sistemas (Fariña & Chilibroste, 2019).

2.2.1. Tipos de encierro

La intensificación en la lechería regional ha llevado a la implementación de diversos sistemas de encierro que responden a la escala y a la capacidad operativa de los establecimientos. En este sentido, el primer nivel de adopción del uso de encierros suelen ser los corrales a cielo abierto (o también llamados *dry-lots* en zonas áridas), seguido por instalaciones de infraestructura más compleja, como es el caso de los galpones de “cama caliente” o *compost barn* (Monge, 2023). Ambos sistemas presentan distintas cualidades y aspectos que merecen atención, teniendo en cuenta las crecientes exigencias del mercado en cuanto a bienestar animal e impacto ambiental.

2.2.1.1. Corrales a cielo abierto

En Uruguay, el proceso de intensificación derivó en la utilización de potreros de sacrificio para reunir a los animales al administrar la suplementación y/o mantenerlos encerrados durante los momentos en que no se pueden acceder a los potreros para pastorear (Chilibroste & Battegazzore, 2019). Estos corrales a cielo abierto resultan fáciles de implementar dado su bajo costo en infraestructura y manejo simple, motivo por el cual también han tenido un interés creciente a nivel internacional (O'Driscoll et al., 2008a; Barnes et al., 2013; USDA-NAHMS, 2016; Salado et al., 2020). El piso es de tierra o tosca, sobre los que se distribuyen los comederos, disponiendo en general de un área de sombra artificial. A nivel internacional su implementación es más común en regiones de clima árido donde son favorecidas las condiciones de piso, si bien el estrés calórico también representa un desafío (Tresoldi et al., 2017). En regiones donde la nieve impide el acceso a las pasturas también ha sido evaluado, pero se requiere el agregado de cama de paja para evitar el congelamiento de pezones (O'Driscoll et al., 2008b; Sjostrom et al., 2019). En Uruguay, la realidad climática significa un desafío en cuanto a la formación de barro, especialmente en invierno, así como también la exposición al estrés calórico durante el verano. La exposición ambiental y condiciones de infraestructura ofrecidas en estos sistemas a cielo abierto podrían provocar estrés y perjuicios en la performance productiva animal.

2.2.1.2. Compost barn

Como una alternativa más amigable con el bienestar animal, surgen sistemas de estabulación denominados *compost barn,* o cama caliente, como es conocido en algunas regiones de Sudamérica. Su popularización a partir del 2001 en Minnesota, y luego en otras regiones, derivó del atractivo por sus beneficios en confort en comparación a los sistemas tradicionales de estabulación como el free-stall, con impactos positivos en salud, reproducción y longevidad de los animales (Barberg et al., 2007). Su adopción en Sudamérica ha sido creciente en los últimos años en sistemas de confinamiento total (Monge et al., 2019; Leso et al., 2020; Monge, 2023). Al momento de iniciar este trabajo doctoral, no se contaba con antecedentes bibliográficos de su uso parcial combinado con pastoreo a nivel comercial. Este sistema se trata de un galpón completamente techado, muchas veces dotado de equipamiento para refrescamiento de los animales con ventilación y aspersores (Leso et al., 2020). Los animales se encuentran libres en una cama de material orgánico, comúnmente de aserrín o chips de madera, que en buenas condiciones confiere un área confortable, mullida y seca, adecuada para el descanso (Endres & Barberg, 2007; Shane et al., 2010; Bewley et al., 2017). Para mantener condiciones de la cama adecuadas, es fundamental la evaporación de la humedad, acumulada con las excreciones de los animales. Para ello, debe ocurrir un proceso de compostaje a través de la fermentación aeróbica (Barberg et al., 2007; Black et al., 2014), que provoca un aumento de temperatura (idealmente por encima de 45°C) en su zona más profunda, promoviendo la evaporación de la humedad, que debería situarse entre 40 y 60% (Black et al., 2013). Esto exige un manejo de movida del material dos veces al día (Barberg et al., 2007) para permitir una correcta oxigenación, el agregado periódico de sustrato, y adecuadas condiciones de ventilación y temperatura del galpón (Black et al., 2014; Leso et al., 2020).

2.3. Respuestas al estrés ambiental

La exposición a condiciones climáticas adversas puede resultar en una cascada de respuestas fisiológicas de estrés, representando un riesgo para la salud y bienestar animal, con consecuencias negativas sobre la performance productiva y reproductiva (Kadzere et al., 2002; De Rensis & Scaramuzzi, 2003; West, 2003; Das et al., 2016). La exposición a lluvias y viento activan el eje hipotálamo-hipófisis-adrenal, con una mayor liberación de cortisol (Fisher et al., 2003; Tucker et al., 2007; Webster et al., 2008), y posibles perjuicios sobre la función inmune (Sapolsky et al., 2000) y reproductiva (Whirledge & Cidlowski, 2010). Tales condiciones son aversivas para el ganado implicando modificaciones comportamentales, con particular impacto en la disminución del consumo y tiempo de descanso durante las precipitaciones (Tucker et al., 2007; Schütz et al., 2010), y por presencia de barro (Chen et al., 2017).

Por otro lado, el estrés calórico, definido como el conjunto de estímulos externos que llevan al aumento no saludable de temperatura corporal del animal (Dikmen & Hansen, 2009), también activan respuestas fisiológicas adaptativas. Está reportado un aumento de la tasa cardíaca y respiratoria, jadeo, transpiración, aumento del consumo de agua y disminución del consumo de alimentos, así como mayor tiempo paradas buscando disipar calor (Das et al., 2016; Polsky & von Keyserlingk, 2017). Para disminuir la producción de calor, adaptaciones metabólicas como una mayor utilización de las proteínas corporales y preservación de las reservas lipídicas también son activadas

(Baumgard & Rhoads, 2013; Gao et al., 2017; Sammad et al., 2020). La vaca lechera posee un complejo mecanismo fisiológico capaz de responder a las condiciones ambientales, al mismo tiempo que está altamente exigido para cumplir con la función para lo cual ha sido seleccionada genéticamente: la producción de leche. Las condiciones de manejo a las que estén expuestas las vacas, sobre todo en los momentos más delicados como su adaptación a la lactancia, definirán la performance y el futuro productivo del animal.

2.4. Época de parición

En sistemas basados en pastoreo, la concentración de partos permite hacer coincidir el ciclo productivo de los animales a los momentos de mayor conveniencia para optimizar recursos. Las principales épocas de parición en dichos sistemas son otoño y primavera (García & Holmes, 2005). En Uruguay, el 65% de los partos se distribuye entre los meses de marzo a septiembre, con picos en los meses de marzo-abril y agosto-septiembre (Chilibroste & Battegazzore, 2019). La concentración de partos se registra principalmente en tambos de mayor escala, mientras que en tambos chicos o de estabulación permanente la parición por lo general se da de forma continua (Pereira et al., 2017).

La parición otoñal (marzo-abril) permite evitar el estrés calórico durante el momento de los servicios, pero el preparto y el postparto temprano aún pueden tener exposición al calor, sobre todo al norte del país (Cruz & Saravia, 2008). Además, el momento de mayor requerimiento energético (lactancia temprana) coincide con la época de mayor probabilidad de escasez de forraje, obligando al uso de encierros y suplementación. El transcurso del invierno y ocurrencia de precipitaciones, promueven la presencia de barro en los lugares de permanencia o tránsito de los animales, amenazando el bienestar animal y humano (Pereira et al., 2017). Por otro lado, los partos más cercanos a la primavera (julio-agosto) tienen la fortaleza de hacer coincidir un mayor número de animales en lactación con la época de mayor disponibilidad de pasturas (Ribeiro et al., 2013; Hennessy et al., 2020). Sin embargo, los animales deberán recibir servicio durante la época que inician los registros de estrés calórico, lo cual puede significar menor éxito en la concepción y mantenimiento de la preñez (De Rensis & Scaramuzzi, 2003; Jordan, 2003). La decisión de la época de concentración de partos impacta en diversos aspectos dentro de los sistemas, incluyendo las condiciones ambientales a las que van a estar expuestos los animales en las distintas etapas de su lactación.

2.5.Período de transición y balance energético negativo

Uno de los momentos más vulnerables en la vida de la vaca lechera ocurre durante la transición de preñada no lactante a no preñada lactante, que se repite a cada nueva lactancia. El periodo de transición se ha definido arbitrariamente como los 21 días previos y posteriores al parto (Grummer et al., 2004). Antes del parto, la capacidad de consumo del animal disminuye, al mismo tiempo que los requerimientos nutricionales aumentan en consecuencia del inicio de la lactogénesis, generando un déficit (Drackley, 1999; Grummer et al., 2004). Este déficit, definido como el BEN, se extiende desde pocos días previos al parto hasta 10 a 12 semanas postparto, alcanzando el nadir alrededor de 15 días postparto (Butler, 2003). En estas circunstancias donde el consumo de energía es menor a la demanda, el organismo del animal se enfrenta a profundas adaptaciones fisiológicas para sostener los procesos prioritarios de mantenimiento y producción.

La regulación aguda de los procesos biológicos y del uso de nutrientes para mantener el equilibrio fisiológico es lo que se conoce como homeostasis (Baumgard et al., 2017). Por otro lado, el cumplimiento de funciones como la producción de leche depende, en segundo lugar, de la orquestación más crónica del flujo de nutrientes y vías de señalización que responden al proceso de homeorhesis o teleoforesis (Bauman & Currie, 1980; Chilliard, 1999; Baumgard et al., 2017). Parte de esta regulación es comandada por hormonas clave del eje somatotrófico (hormona del crecimiento - GH, factor insulino-símil 1 -IGF-1-, e insulina), que coordinan las adaptaciones para mantener la homeostasis metabólica y garantizar la partición de nutrientes para la producción de leche (Bell, 1995; Kim, 2014).

Luego del parto, las concentraciones de IGF-1 se reducen a pesar de las altas concentraciones de GH, que estimulan su producción. Esto ocurre por la menor abundancia de su receptor GHR1A en hígado en consecuencia del BEN y las bajas concentraciones de insulina que estimulan su expresión (Butler et al., 2003), resultando en el desacople del eje somatotrófico (Lucy et al., 2001; Kim, 2014). Las bajas concentraciones de IGF-1 no ejercen la usual retroalimentación negativa hipofisiaria que controla la secreción de GH, por lo que éstas aumentan más en el período. Las altas concentraciones de GH estimulan la lipólisis. Los triacilglicéridos almacenados en el tejido adiposo son hidrolizados, liberando ácidos grasos no esterificados (NEFA), que continúan a la ruta de la β-oxidación en hígado y músculo, formando acetil-CoA (Nelson & Cox, 2013). Frente a altos niveles de producción de acetil-CoA, parte del mismo no logra ingresar al ciclo de Krebs siendo redirigido a la vía cetogénica, formando cuerpos cetónicos como el β-hidroxibutirato (BHB), acetoacetato y acetona en el hígado (Nelson & Cox, 2013). La incapacidad de ingreso de acetil-CoA al ciclo de Krebs se debe a la limitada disponibilidad de oxaloacetato para unirse, el cual está siendo redirigido a la vía neoglucogénica en hígado.

También estimulada por la GH, la neoglucogénesis es prioritaria para la lactogénesis. La degradación de proteínas tisulares que aportan esqueletos carbonados de ciertos aminoácidos también colaboran a la formación de glucosa que posteriormente formará lactosa en glándula mamaria (Bauman & Currie, 1980; Herdt, 2000). Como mecanismo de ahorro, el consumo de glucosa por parte de los tejidos periféricos se ve limitado, producto de la resistencia a la insulina (Robinson & Williamson, 1980). Durante el pico de lactancia disminuye el contenido de transportadores de glucosa dependientes de insulina (GLUT4) en músculo esquelético y tejido adiposo, que también presenta menor expresión de GLUT1. Este transportador, independiente de insulina, es el principal responsable por el ingreso de glucosa en glándula mamaria, pasando de ser casi indetectable en el periodo seco a multiplicar en varias veces su expresión en lactancia temprana (De Koster & Opsomer, 2013). De esta manera, el 50 a 85% del consumo total de glucosa del cuerpo se da por parte de la glándula mamaria, amplificando la demanda en 2,5 veces en la tercer semana de lactancia comparado al periodo seco (De Koster & Opsomer, 2013).

Adicionalmente, la homeorhesis también es comandada por las funciones de crecimiento (Bauman et al., 1982). Vacas primíparas que inician su lactancia sin haber completado su crecimiento y desarrollo, presentan diferencias endócrino-metabólicas con las vacas multíparas (Wathes et al., 2006). Insulina e IGF-1 también estimulan el crecimiento, con perfiles de IGF-1 consistentemente más altos en vacas primíparas respecto a multíparas y una partición de nutrientes para producción de leche limitada en la categoría más joven (Wathes et al., 2006, 2007). Sin embargo, si bien en condiciones pastoriles las concentraciones iniciales de IGF-1 en primíparas son mayores que en multíparas, el desacople (es decir, la caída brusca en las concentraciones de IGF-1 en el período de transición) es mayor en primíparas que en multíparas (Meikle et al., 2004; Adrien et al., 2012). Esto se refleja en las reservas corporales evaluadas subjetivamente a través de la condición corporal, donde las primíparas a pesar de llegar con un nivel adecuado al parto (entre 3 a 3,25; Roche et al., 2009), podrían presentar una caída más pronunciada luego del mismo según las condiciones de manejo a las que estén sometidas (Meikle et al., 2004, 2018). En sistemas pastoriles, ambas categorías son usualmente manejadas juntas, y factores de dominancia a la hora de disputar el alimento podrían ser perjudiciales para las primíparas (Meikle et al., 2018).

Condiciones menos restrictivas, como alimentación *ad libitum* con TMR podrían atenuar la magnitud de la movilización de reservas y balance energético negativo al compararse con sistemas basados en pastoreo (Astessiano et al., 2015; Cardoso et al., 2020). De hecho, vacas alimentadas con TMR presentaron mayores concentraciones plasmáticas de insulina e IGF-1 comparado a vacas en pastoreo con alta asignación de forraje y suplementación (Meikle et al., 2013). Las diferencias se han atribuido a la mayor densidad en nutrientes en la TMR, así como también al ahorro del costo energético por la caminata y actividad de pastoreo respecto a los sistemas pastoriles (Kolver & Muller, 1998; Talmón et al., 2025). Por lo tanto, las estrategias basadas en el manejo alimenticio poseen el potencial de mejorar la adaptación metabólica a la lactancia, que sostiene la base de la performance productiva y reproductiva de los animales (Butler, 2014; Cardoso et al., 2020).

2.6. Balance energético y función reproductiva postparto

La sustentabilidad del sistema lechero depende en gran medida de la eficiencia reproductiva. Lograr la preñez alrededor de los 3 meses de lactación es clave en potenciar el número de lactancias y por ende de picos de producción en la vida de la vaca, mejorando el margen económico (Overton & Eicker, 2025). El primer desafío para una correcta fertilidad es superar el periodo de anestro postparto. Vacas que logran expresar celo y ovular más temprano luego del parto, tienen un menor intervalo parto-concepción y menor probabilidad de ser descartadas del rodeo (Darwash et al., 1997; Rhodes et al., 2003). Mas aun, las vacas que logran expresar celo antes de los 30 días postparto requieren menos servicios para lograr la concepción y tienen mayor tasa de no retorno (Thatcher & Wilcox, 1973). Aspectos definitorios del tiempo al reinicio de la actividad ovárica son la condición corporal con que llegan al parto y el grado de movilización postparto (Roche et al., 2009; Bedere et al., 2018), así como enfermedades del periparto, consumo de energía y partición de nutrientes hacia la producción de leche (Rhodes et al., 2003).

De manera general se acepta que la magnitud del BEN es un regulador clave del estatus reproductivo (Butler & Smith, 1989). Ambas hormonas, insulina e IGF-1, ejercen *feedback* positivo sobre el eje reproductivo tanto a nivel central como periférico (Meikle et al., 2018), y se asocian con el tiempo de reinicio de la ciclicidad ovárica (Rhodes et al., 2003). Factores como alteración de la pulsatilidad y respuesta a la LH, desarrollo folicular y producción de estradiol podrían estar involucrados en su vinculación (Diskin et al., 2003). Vacas lecheras clasificadas como en buen estatus metabólico (alta glucosa, bajo NEFA y bajo BHB), tuvieron mayor probabilidad de presentar cuerpo lúteo a las 3 semanas postparto que vacas con peor estatus metabólico (Rojas Canadas et al., 2020). Además, la paridad y edad de las vacas también impacta en el tiempo al reinicio a la ciclicidad. Existen evidencias de que vacas primíparas de 2 años poseen un retraso al reinicio en relación a las de 3 años o a multíparas (Rhodes et al., 2003; Rojas Canadas et al., 2020), aun cuando su estatus metabólico suele ser mejor que el de multíparas (Rojas Canadas et al., 2020). Estos autores sugieren que las primíparas parecen ser más sensibles a las señales endócrino-metabólicas durante el periodo de transición, aumentando el tiempo para el reinicio de la ciclicidad, mientras priorizan la finalización de su desarrollo corporal (Rojas Canadas et al., 2020). Además, la adaptación al parto y al nuevo manejo de ordeñe también podrían tener incidencia en esta respuesta (Eicher et al., 2007; Ruprechter et al., 2020). No obstante, en condiciones en que el animal no esté bajo estrés nutricional y haya parido con adecuada condición corporal, es posible lograr la ovulación del primer folículo dominante alrededor de los 15 días postparto en gran parte de las vacas (Crowe et al., 2014).

Por otro lado, el desafío de mantener la gestación luego de lograr la fertilización con gametos de buena calidad, es hoy en día uno de los focos de estudio de la comunidad científica internacional. La mayoría de las pérdidas de preñez ocurre previo al periodo de implantación (día 19), a partir de cuando la nutrición hemotrófica se inicia (Binelli et al., 2022). En este periodo, el desarrollo del embrión depende del histotrofo, definido por el conjunto de moléculas secretadas por el endometrio o transportadas al lumen que actúan como combustible y cofactores de diversos procesos (Roberts & Bazer, 1988; Binelli et al., 2022). El día 7 del ciclo estral es un momento importante de remodelación uterina e inicio de la secreción del histotrofo (Moran et al., 2017), fundamental para sostener el embrión luego de la eclosión de la zona pelúcida, hasta que se establezca la conexión hemática con la madre. La mayoría de las pérdidas embrionarias ocurre ya en la primer semana de gestación (Diskin et al., 2006; Berg et al., 2022). Se ha cuantificado que dichas pérdidas pueden rondar entre 10 y 50% de las fallas, y se ha atribuido como una de las principales causas los problemas metabólicos postparto (Wiltbank et al., 2016).

La adaptación metabólica para sustentar la producción de leche podría representar un desafío en la compatibilidad para mantener la preñez, en un contexto en que los servicios se realizan próximo al pico de lactación. Las causas de las fallas podrían ser multifactoriales, con perjuicios de mediano plazo del balance energético sobre la calidad de los ovocitos (Britt, 1992; Leroy et al., 2008), como sobre la receptividad del tracto reproductivo (Valour et al., 2013; Tinning et al., 2023). Se ha sugerido que el endometrio podría censar el estatus metabólico del animal adaptando su fisiología (Meikle et al., 2018). Experimentos en los que se realizó transferencia de embriones al día 7 - de manera a evadir ruidos atribuibles a calidad de gametos-, han demostrado menores tasas de preñez en receptoras en lactación respecto a vacas secas o vaquillonas, sugiriendo que las condiciones del endometrio para albergar el embrión en las primeras serían subóptimas (Hasler, 2001; Chagas e Silva et al., 2002; Wilson et al., 2006). La lactación parecería generar disrupciones en la regulación de la función inmune, clave para la tolerancia del embrión semialogénico, así como también en los mecanismos de homeostasis de la glucosa en el endometrio (Cerri et al., 2012). Un experimento donde se recuperaron embriones al día 7 post inseminación de las mismas vacas luego del primer celo (~ 40 días en leche -DIM-) vs el tercer celo (~ 80 DIM), reveló que si bien el porcentaje de recuperación fue similar (~70%), había una mayor proporción de embriones de excelente y buena calidad (Grados 1 y 2) en el tercer celo comparado con el primer celo (79% vs. 56%, respectivamente; Aranciaga et al., 2023). Las concentraciones circulantes de NEFA y BHB fueron menores en el tercer vs el primer celo, reflejando un mejor balance energético a medida que transcurrieron los días (Aranciaga et al., 2023).

Vacas en BEN más severo presentan evidencia de mayor grado de inflamación endometrial a los 14 días postparto, con expresión diferencial de genes implicados a la respuesta inmune y remodelación tisular (Wathes et al., 2009). Estas funciones parecerían ser claves, dado que vacas con mayor mérito genético para fertilidad presentan a nivel del endometrio una menor expresión de genes promotores de inflamación, y una mayor expresión de genes relacionados a componentes de citoesqueleto y transporte de iones al día 7 del ciclo estral (Moran et al., 2017). El grado de BEN y los indicadores endócrino-metabólicos, afectados por el manejo de la alimentación (restricción leve de 20% de los requerimientos vs control), también alteraron la expresión génica del tracto reproductivo al momento de los servicios (Valour et al., 2013). Las concentraciones de BHB, insulina e IGF-1, pero también su cinética a lo largo de todo el periodo postparto, se correlacionaron con genes relacionados a reparación tisular a nivel de estructura, metabolismo e inmunidad (Valour et al., 2013). Entre los DEGs, *FABP5*, que está relacionado al metabolismo lipídico, estuvo subexpresado tanto en oviducto como endometrio en vacas en BEN más pronunciado, mientras que en endometrio también se encontró una subexpresión de *RBM23*, involucrado en la transcripción de genes que responden a esteroides. Antecedentes nacionales en vacas primíparas sugieren una expresión génica endometrial más favorable para el éxito de la preñez en animales con mejor nivel nutricional tanto en TMR como con alta asignación de forraje más PMR, respecto a menor asignación de forraje (Astessiano et al., 2017). Las dietas que maximizaron la ingesta de energía podrían favorecer el desarrollo embrionario dado su mayor expresión en genes que transcriben receptores de progesterona y adiponectina, así como los relacionados al sistema IGF (*IGF1, IGF2, IGFR, IGFBP-3,4,5*; Astessiano et al., 2017). Estos indicadores podrían explicar el vínculo entre performance reproductiva y balance energético, a su vez dependiente del nivel nutricional de los animales (Wathes et al., 1998; Valour et al., 2013; Astessiano et al., 2017).

A pesar de la abundante literatura sobre el perfil de metabolitos en la circulación general durante el balance energético negativo, el conocimiento sobre como el ambiente uterino se ve afectado en esta circunstancia aún es limitado. El estatus metabólico podría afectar el perfil de moléculas presentes en el fluido uterino, ya sea por lo que llega mediante circulación directa, o por las rutas activadas en el propio endometrio mediante esa expresión diferencial de genes para su producción y/o transporte (Fair, 2014; Aranciaga et al., 2021). Acceder al perfil metabolómico en el tracto reproductor con herramientas como la resonancia magnética nuclear, tiene un potencial promisor para comprender la fisiología que lleva al éxito reproductivo (Baskind et al., 2011). Caracterizaciones del fluido uterino de vaquillonas preñadas clasificadas por fertilidad indican diferencias en el metaboloma relacionadas a vías de metabolismo energético y aminoacídico (Moraes et al., 2020), por lo que se podría esperar que el metabolismo a nivel sistémico pudiera impactar su capacidad reproductiva. El fluido uterino de vacas preñadas más temprano respecto al parto (~40 DIM) presentaba un perfil metabolómico compatible con mayor actividad metabólica y reacciones oxidativas, respecto al fluido de las mismas vacas preñadas nuevamente más tarde (~d79 DIM; Aranciaga et al., 2023). Esto podría sugerir un efecto de la magnitud de balance energético negativo sobre el ambiente uterino, y estar asociado con los hallazgos de peor calidad de embriones obtenidos de la primer preñez respecto a la siguiente, como mencionado anteriormente (Aranciaga et al., 2023). Sin embargo, para nuestro conocimiento no existen estudios que hayan evaluado el impacto del manejo nutricional sobre el balance energético al inicio de lactancia y su efecto residual sobre el metaboloma uterino al momento de iniciar los servicios.

3. PLANTEAMIENTO DEL PROBLEMA

La vaca lechera moderna está sometida a una presión metabólica intensa para sostener altos niveles de producción de leche. Los sistemas lecheros han presentado un alto grado de intensificación, con modificaciones en el ambiente productivo y alimentación, pero el conocimiento sobre el impacto de estas condiciones en la adaptación metabólica de los animales al inicio de la lactancia aún es limitado. Del éxito con que el animal logre dicha adaptación, dependerá su performance productiva y reproductiva, y por tanto su permanencia en el rodeo, incidiendo sobre la rentabilidad de los sistemas.

Los sistemas actuales que combinan pastoreo con suplementación en encierros a cielo abierto, podrían someter a los animales a condiciones de estrés ambiental, pero no se conoce su impacto sobre la adaptación metabólica y performance productiva animal. El creciente interés por los sistemas más amigables desde el punto de vista de confort animal como el *compost barn,* representa una alternativa para evitar estas condiciones de estrés. No obstante, su uso parcial en combinación con pastoreo no había sido explorado hasta la ejecución de este trabajo. Más aún, en Uruguay tampoco se contaba con antecedentes de su uso en condiciones de estabulación total, que dieran cuenta de su potencial en cuanto a performance productiva. Dados los desafíos que podrían implicar estos sistemas intensivos de producción de leche, surge la necesidad de responder si las condiciones de infraestructura durante el encierro, así como diferencias en el sistema de alimentación, podrían afectar la adaptación metabólica a la lactancia y sus consecuencias en producción y ciclicidad ovárica.

Por otro lado, si bien se conoce que el manejo nutricional presenta un gran potencial de impacto en el balance energético (Cardoso et al., 2020), los estudios de los actuales sistemas que combinan pastoreo y suplementación con PMR son escasos (Bargo et al., 2002; Mendoza et al., 2016a; Salado et al., 2018) y pocos se han enfocado en el periodo más crítico de lactancia temprana (Astessiano et al., 2015). Existe evidencia de que la magnitud de balance energético negativo sería mayor en sistemas pastoriles comparado a sistemas confinados, aun cuando la participación de la pastura en la dieta da cuenta de solo un tercio de la ingesta (Astessiano et al., 2015). Esto lleva a pensar que un manejo estratégico de corto plazo, contemplando la etapa más crítica - postparto - del periodo de transición, podría tener un impacto positivo sobre la adaptación metabólica, con potenciales beneficios sobre la performance productiva y reproductiva, sin perder los beneficios de la cosecha directa de forraje -alimento más económico- posteriormente. La literatura contemplando este tipo de estrategia es igualmente escasa, e indicarían que la estabulación durante las primeras semanas de lactación tendría beneficios sobre indicadores metabólicos respecto a vacas bajo pastoreo desde el parto, pero sin diferencias o de baja magnitud sobre la producción de leche o reinicio de ciclicidad ovárica (Al Ibrahim et al., 2013; Brady et al., 2021). Sin embargo, no se evaluaron diferencias en cuanto a la paridad, teniendo en cuenta que el potencial de partición de nutrientes está asociado a la categoría animal (Wathes et al., 2006). Más aún, no se encontraron antecedentes que evalúen el efecto residual de este tipo de manejo sobre el ambiente uterino al momento de iniciar los servicios.

4. HIPÓTESIS

I. En sistemas mixtos que combinan pastoreo y suplementación, el encierro parcial en galpón de *compost* se asociará con mejores indicadores productivos y reproductivos respecto al encierro parcial a cielo abierto, lo que se verá reflejado en los perfiles endócrino-metabólicos durante la lactancia temprana. A su vez, ambos sistemas mixtos tendrán una peor performance productiva y estatus energético (en términos de indicadores endócrino-metabólicos) que sistemas en estabulación completa en galpón de *compost*, alimentados *ad libitum* con TMR (**Artículo I**).

II. Manejos estratégicos de estabulación alimentados con TMR *ad libitum* durante los primeros 21 DIM, proporcionan una mejor adaptación endócrino-metabólica a la lactancia, asociado a mayor producción, un reinicio de la ciclicidad ovárica postparto más rápido y mejor ambiente uterino (en términos de metaboloma y transcriptoma), en comparación con sistemas en pastoreo + PMR implementados inmediatamente luego del parto. A su vez, se hipotetiza que las respuestas al manejo diferencial dependerán de la categoría animal (primíparas vs multíparas) (**Artículos II y III**).

5. OBJETIVOS

5.1. Objetivo general

El objetivo general de este proyecto doctoral es contribuir al conocimiento del manejo estratégico (con foco en la alimentación e infraestructura) de la vaca lechera durante la lactancia temprana para facilitar la adaptación metabólica a la lactancia, mejorando los parámetros productivos y reproductivos, en sistemas de alimentación en base a pasturas.

5.2. Objetivos específicos

Determinar si el ambiente productivo y/o manejo alimenticio durante la lactancia temprana afectan:

I. La producción de leche individual, producción de sólidos y condición corporal durante la lactancia temprana (**Artículos I y II**).

II. El estatus endócrino-metabólico medido a través de las concentraciones séricas de ácido grasos no esterificados (NEFA), β-hidroxibutirato (BHB), colesterol, factor de crecimiento similar a la insulina-1 (IGF-1), insulina, y la probabilidad de reinicio de ciclicidad ovárica durante la lactancia temprana (**Artículos I y II**).

III. El metaboloma sérico a los 21 y 60 DIM de vacas estabuladas por 21 días luego del parto y vacas en pastoreo con suplementación desde el parto (**Artículo II**).

IV. La concentración sérica de progesterona durante la fase lútea temprana al momento de iniciar los servicios (60 DIM) de vacas estabuladas por 21 días luego del parto y de vacas en pastoreo con suplementación desde el parto (**Artículo III**).

V. La expresión génica y el metaboloma endometrial en la fase lútea temprana al momento de iniciar los servicios (60 DIM) de vacas estabuladas por 21 días luego del parto y de vacas en pastoreo con suplementación desde el parto (**Artículo III**).

6. Artículo I:

“Adaptación metabólica a la lactancia de vacas lecheras en dos condiciones contrastantes de infraestructura durante el encierro con pastoreo o en estabulación completa”.

“Metabolic adaptation to lactation of dairy cows in two contrasting facilities involving partial confinement plus grazing or total confinement”.

6.1.Objetivos específicos

Determinar el efecto del tipo de encierro parcial (galpón de *compost* vs cielo abierto) en sistemas de base pastoril combinados con suplementación, sobre los perfiles endócrino-metabólicos durante la lactancia temprana, parámetros productivos y ciclicidad ovárica postparto. Comparar las variables mencionadas para ambos grupos con vacas en sistema de estabulación completa en galpón de *compost*, alimentadas *ad libitum* con TMR (**Artículo I**).

6.2.Materiales y métodos del Experimento I (Artículo I)

Este experimento fue realizado en la Estación Experimental Dr. Mario A. Cassinoni, Paysandú, y fue aprobado por el Comité de Ética en el Uso de Animales de Experimentación (ID 682- Exp. 020300- 000602-18). Se utilizaron vacas lecheras de la raza Holstein multíparas que parieron en otoño (n = 36) y primavera (n = 48). Los animales fueron bloqueados según número de lactancias, fecha probable de parto, peso vivo y condición corporal, y se distribuyeron en los siguientes tres tratamientos desde el parto hasta los 90 días postparto: pastoreo y suplementación con PMR en corrales a cielo abierto (OD-GRZ), pastoreo y suplementación con PMR en galpón de *compost* (CB-GRZ), estabulación completa con TMR *ad libitum* en galpón de *compost* (CB-TMR). La representación esquemática de los tratamientos se ilustra en la Figura 1. Las condiciones de infraestructura de los encierros a cielo abierto (Fig. 2) o galpón de *compost* (Fig. 3) eran contrastantes, de manera a responder la hipótesis 1. Los tratamientos en pastoreo se manejaron en una plataforma de pastoreo de alta carga (2,5 vacas en ordeñe/hectárea). Se asignaban parcelas semanales independientes contiguas para cada tratamiento, con el mismo recurso forrajero. La asignación de forraje se ajustaba semanalmente acorde a la tasa de crecimiento y condiciones de la planta (número de hojas o nudos) y con el objetivo de mantener un stock de forraje de 1800 kgMS/ha en la plataforma. Asumiendo una utilización de forraje de 50%, se ajustaba la cantidad de PMR a ser suplementada para ambos tratamientos. La TMR y PMR estaban compuestas de la misma mezcla de ingredientes, y la oferta para el grupo CB-TMR se ajustaba de manera a obtener un consumo *ad libitum* con un 5% de rechazo. Detalles de los recursos forrajeros utilizados, ingredientes de la dieta mezcla, composición química de pastura y dieta mezcla, bien como la estimación de consumo de TMR/PMR y pastura por mes, se encuentran en detalle en el artículo I. Se determinó mediante medidas repetidas la producción de leche, sólidos totales en leche (TMS), condición corporal, las concentraciones de NEFA, BHB, colesterol, insulina e IGF-1, así como también la proporción de vacas con cuerpo lúteo. Otros detalles respecto al manejo, alimentación y determinaciones se describen en el artículo en anexos.

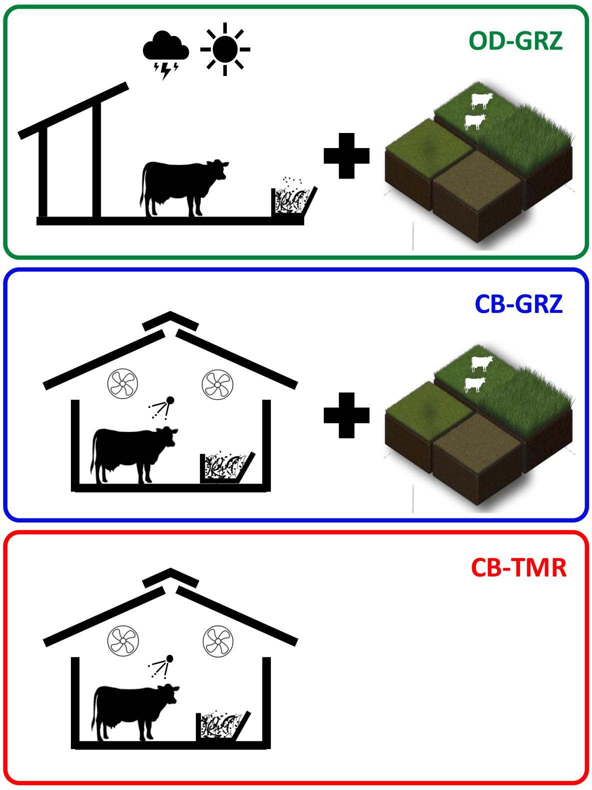

Figura 1. Esquema representativo de los tratamientos en pastoreo y suplementación con PMR en corrales a cielo abierto (OD-GRZ), pastoreo y suplementación con PMR en galpón de *compost* (CB-GRZ), estabulación completa con TMR *ad libitum* en galpón de *compost* (CB-TMR).

Figura 2. Imagen aérea del encierro a cielo abierto (A), sombras artificiales (B) y estado del piso luego de lluvias (C).

Figura 3. Imagen aérea del galpón de *compost* (A), animales echados en la cama de *compost* (B) y sistema de refrescamiento con aspersores y ventiladores (C).

6.2.1. Análisis estadístico

Cada estación (otoño y primavera) fue analizada por separado. Los datos se analizaron mediante un modelo lineal generalizado mixto (GLIMMIX, SAS Studio®) con medidas repetidas en el tiempo, teniendo en cuenta la distribución de los datos. Se consideró como efectos fijos el tratamiento, los días en leche (DIM) y su interacción, y como efecto aleatorio el bloque. Las comparaciones post hoc se realizaron con la prueba de Tukey–Kramer. Se consideró significancia cuando $P \leq 0.05$ y tendencia cuando $0.05 < P \leq 0.10$.

6.3. Principales resultados Experimento I (Artículo I)

En ambas estaciones de parto la producción de leche (Fig. 4 A y B) y los TMS no difirieron entre OD-GRZ y CB-GRZ, pero fueron mayores en CB-TMR (P < 0,0001). En otoño, las concentraciones de NEFA y BHB no se vieron afectadas por los tratamientos, pero la concentración del colesterol aumentó más rápido en CB-TMR (P = 0,05). En primavera, las concentraciones de NEFA y colesterol no se vieron afectadas por el tratamiento, pero BHB fue mayor en OD-GRZ a los 60 DIM en comparación a CB-TMR y a ambos CB-GRZ y CB-TMR a los 90 días (Fig. 4 D). Las concentraciones de IGF-1 e insulina no difirieron entre los tratamientos en otoño, pero en primavera, el IGF-1 fue mayor en CB-TMR (P < 0,0001; Fig. 4 F). La proporción de vacas con cuerpo lúteo no fue diferente entre los tratamientos en pastoreo, pero ambos fueron mayores que CB-TMR hasta los 40 DIM en otoño (P = 0,0489; Fig. 5 A) y durante todo el estudio en primavera (P = 0,0285; Fig. 5 B).

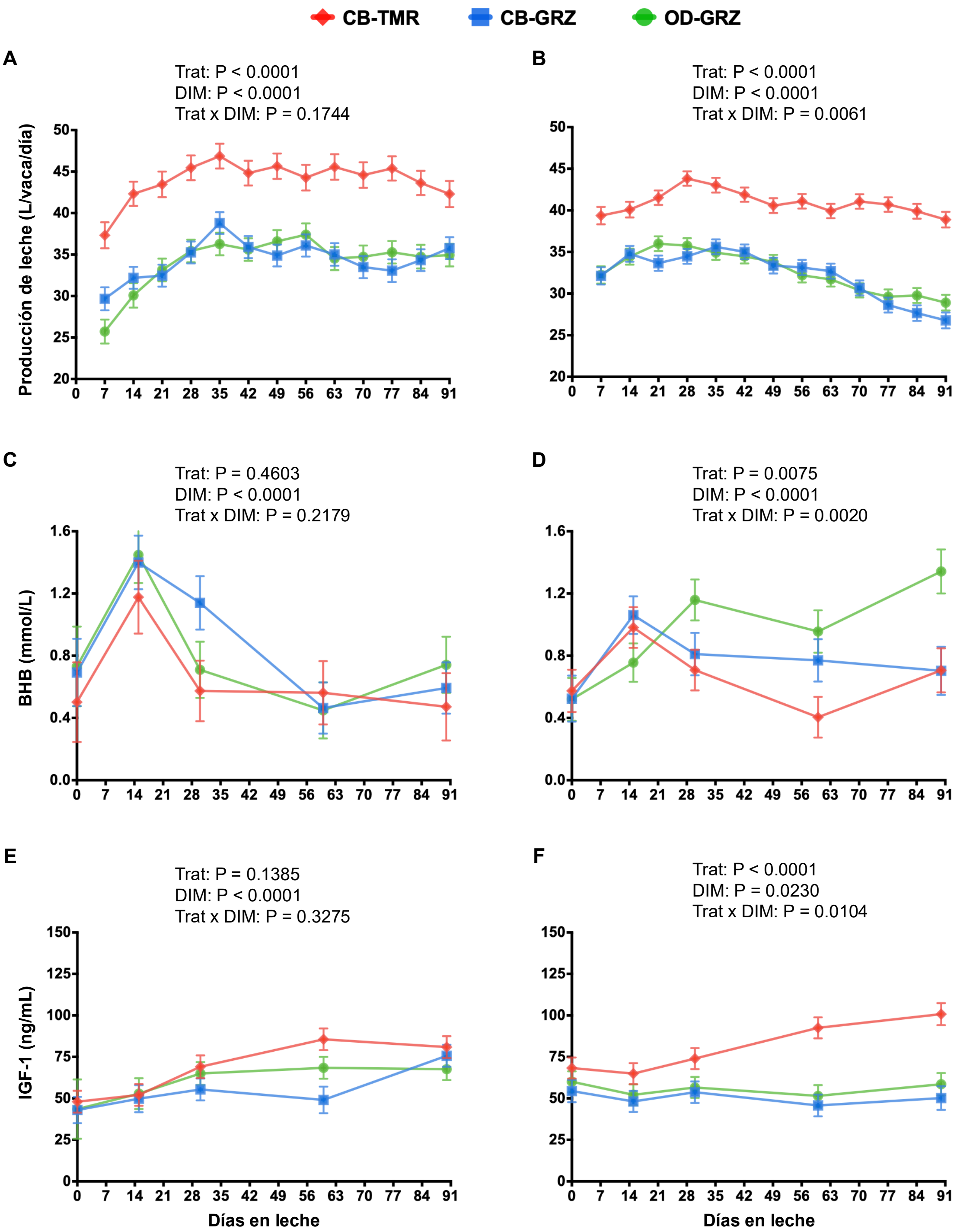

Figura 4. Producción de leche, concentraciones séricas de β-hidroxibutirato (BHB) y factor de crecimiento similar a la insulina 1 (IGF-1) en vacas paridas en otoño (izquierda) y primavera (derecha) en los tratamientos de pastoreo y suplementación en encierro a cielo abierto (OD-GRZ), pastoreo y suplementación en galpón de *compost* (CB-GRZ), y estabulación total con TMR *ad libitum* en galpón de *compost* (CB-TMR), durante los primeros 90 días en leche.

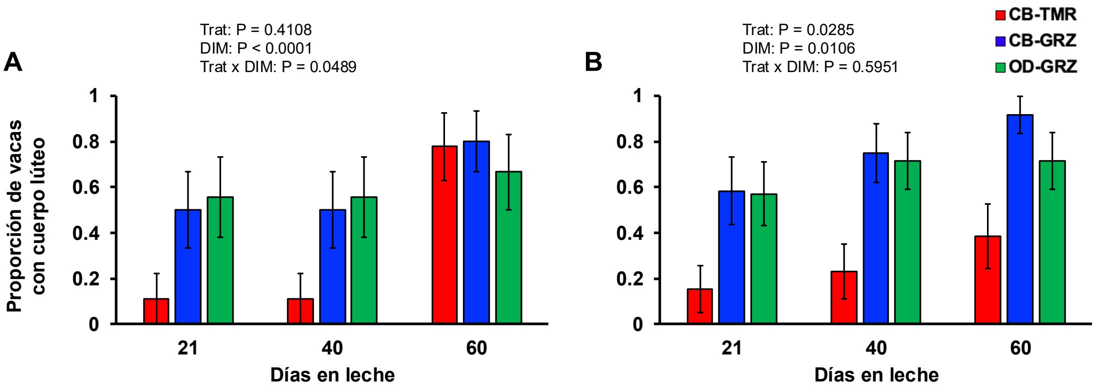

Figura 5. Proporción de vacas con cuerpo lúteo a los 21, 40 y 60 días en leche, en los tratamientos de pastoreo y suplementación en encierro a cielo abierto (OD-GRZ), pastoreo y suplementación en galpón de *compost* (CB-GRZ), y estabulación total con TMR ad libitum en galpón de *compost* (CB-TMR), paridas en otoño (izquierda) y primavera (derecha).

7. Artículos II y III

Manejo alimenticio estratégico durante los primeros 21 días postparto: impacto sobre la adaptación metabólica a la lactancia, reinicio de ciclicidad y ambiente uterino al momento de iniciar los servicios

Art. II: ***Feeding strategies during fresh cow period in pasture-based dairy systems: metabolic adaptation to lactation and resumption of ovarian cyclicity in primiparous and multiparous cows***

Art. III: ***Is there any residual effect of differential feeding strategies during the fresh period on endometrium transcriptomics and metabolomics at the end of the voluntary waiting period in the dairy cow?***

7.1.Objetivos específicos

Determinar el efecto de un manejo diferencial estratégico basado en estabulación con TMR *ad libitum* durante los primeros 21 DIM vs sistemas en pastoreo + suplementación con PMR implementados inmediatamente luego del parto, sobre los parámetros productivos, indicadores endócrino-metabólicos y metabolómica sérica, reinicio de la ciclicidad ovárica postparto y metaboloma y transcriptoma uterino en vacas primíparas y multíparas (Artículos II y III).

7.2. Materiales y métodos del Experimento II (Artículo II y III)

Este experimento fue realizado en la Estación Experimental Dr. Mario A. Cassinoni, Paysandú, y fue aprobado por el Comité de Ética en el Uso de Animales de Experimentación (ID 1344 - Exp. 020300-501632-21). Se utilizaron vacas primíparas (PP) y multíparas (MP) paridas en primavera, bloqueadas según número de lactancias, fecha probable de parto, peso vivo y condición corporal. Los bloques se dividieron en los siguientes dos tratamientos: **T0** (PP: n=8, MP: n=12), en el que inmediatamente luego del parto las vacas fueron manejadas en un sistema con acceso al pastoreo en un turno y suplementación con PMR en el otro turno; y **T21** (PP: n=8, MP: n=12), en el que durante los primeros 21 DIM las vacas estuvieron estabuladas en galpón con TMR *ad libitum* y a partir de los 22 DIM pasaron a ser manejadas junto a las vacas T0. Este manejo se realizó hasta los 63 DIM aproximadamente (Figura 6). La cantidad de TMR ofrecida a T21 se ajustó de manera a obtener un consumo *ad libitum* (aproximadamente 10 % de rechazo). Para las vacas T0, la PMR fue suministrada de forma a complementar el consumo de pasto, dependiendo de la asignación semanal de pastura. La oferta diaria de forraje fue al menos tres veces superior al consumo de materia seca esperado (25 a 30 kg de MS/vaca/día a nivel del piso). Junto al artículo II se detalla información referente a los recursos forrajeros utilizados, ingredientes de la dieta mezcla, composición química de pastura y dieta mezcla, y cantidades ofertadas.

Se determinó la producción de leche, TMS, condición corporal, las concentraciones séricas de NEFA, BHB, colesterol, insulina, IGF-1, así como progesterona para determinar probabilidad de reinicio de ciclicidad. Además, se tomaron muestras de suero el último día de manejo diferencial (21 DIM) y a los 60 DIM para análisis de metabolómica a través de resonancia magnética nuclear no dirigida. A los 40 días posparto se realizó una sincronización y manejo específico para determinar el efecto de los tratamientos sobre el metaboloma y transcriptoma uterino a los 60 días posparto en vacas primíparas y multíparas (esta sección se describe en detalle más adelante).

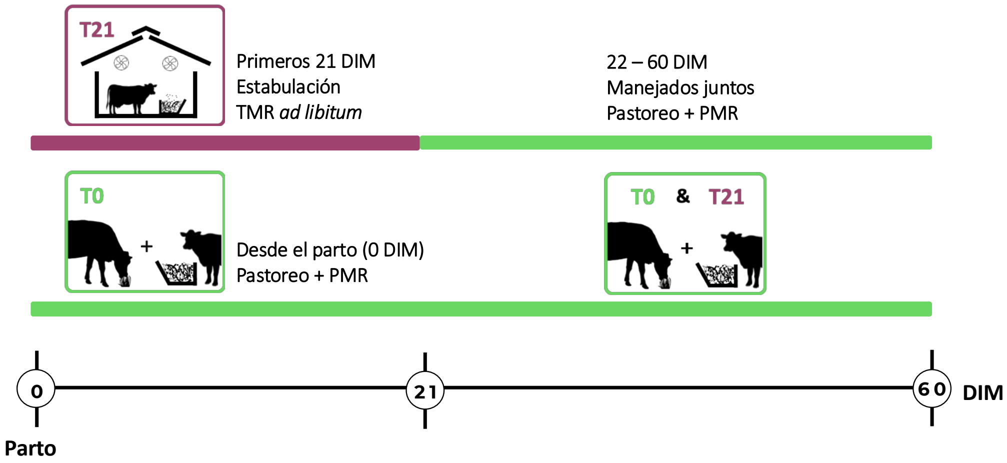

Figura 6. Esquema representativo de los tratamientos en pastoreo y suplementación con PMR desde el parto (T0), y estabulación completa con TMR *ad libitum* durante los primeros 21 días en leche (DIM), seguido por pastoreo y suplementación con PMR (T21).

7.2.1. *Análisis estadístico*

Los datos de producción de leche, TMS, condición corporal, metabolitos y hormonas en suero, se analizaron mediante un modelo lineal generalizado mixto (GLIMMIX, SAS Studio®) con medidas repetidas. Se consideró como efectos fijos el tratamiento, la paridad, los DIM y sus interacciones, y como efecto aleatorio el bloque. Cuando las interacciones fueron no significativas, las mismas se removieron del modelo. Las comparaciones post hoc se realizaron con la prueba de Tukey–Kramer. Se consideró significancia cuando $P \leq 0{,}05$ y tendencia cuando $0.05 < P \leq 0{,}10$. El análisis de la probabilidad de reinicio de ciclicidad ovárica se realizó mediante modelos de regresión de riesgos proporcionales de Cox (procedimiento PHREG; SAS Studio®), incluyendo como efectos fijos el tratamiento y la paridad. Cuando significativos, los resultados se presentan como un cociente de riesgos instantáneos (HR) establecido para el grupo de referencia, donde un HR > 1 significa que un evento ocurre antes, mientras que un HR < 1 significa que un evento ocurrirá más tarde (Cox, 1972). Para la metabolómica sérica se realizaron análisis multivariados, incluyendo el análisis de componentes principales (PCA) y el análisis discriminante de mínimos cuadrados parciales ortogonales (OPLS-DA), como descripto por López Radcenco (2021). Como complemento, se realizaron análisis univariados para cada metabolito con un modelo lineal generalizado mixto (GLIMMIX, SAS Studio®) como descripto anteriormente.

7.3. Principales resultados incluidos en artículo II

La producción de leche estuvo afectada por la triple interacción entre tratamiento, paridad y DIM ($P < 0{,}0001$). Durante el período de manejo diferencial, no se observaron diferencias en la producción de leche entre tratamientos en vacas primíparas (Fig. 7A), mientras que las vacas multíparas T21 produjeron más leche que las vacas T0 (Fig. 7B). Tras el cambio a pastoreo, las vacas primíparas T21 presentaron una menor producción de leche que las vacas primíparas T0 entre los 28 y los 42 DIM, y posteriormente la producción de leche convergió entre tratamientos. En las vacas multíparas T21, la producción de leche disminuyó tras el cambio de manejo, alcanzando niveles comparables a los de las vacas multíparas T0 y se mantuvo estable hasta el final del estudio. No hubo efecto de tratamiento sobre los días al reinicio de ciclicidad ovárica, observándose una menor razón de riesgo de reiniciar la ciclicidad en primíparas que en multíparas (HR = 0,42; $P = 0{,}0380$). Las vacas multíparas exhibieron un reinicio de ciclicidad más temprano (28,5 ± 2,2 días) que las primíparas (36,4 ± 3,8 días).

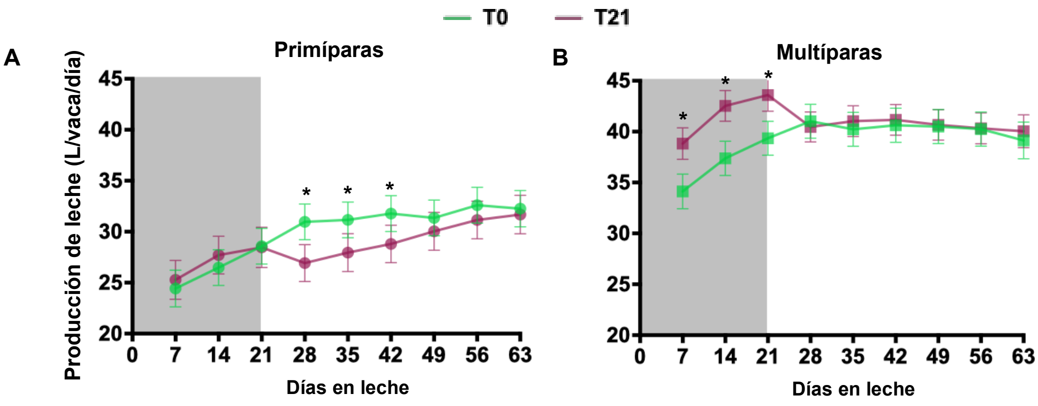

Figura 7. Producción de leche de vacas primíparas (izquierda) y multíparas (derecha) en los tratamientos de pastoreo y suplementación desde el parto (T0) y en estabulación estratégica con TMR *ad libitum* durante los primeros 21 días en leche, seguido por pastoreo y suplementación (T21), hasta los 63 días en leche.

Las concentraciones de NEFA estuvieron afectadas por la triple interacción de tratamiento, paridad y DIM (P = 0,0376). En vacas primíparas T0, las concentraciones de NEFA fueron mayores que en vacas primíparas T21 durante los primeros 21 DIM (Fig. 8A). Por otro lado, en las vacas multíparas el tratamiento no mostró diferencias en las concentraciones de NEFA hasta los 15 DIM, después de lo cual las vacas multíparas T21 exhibieron una disminución más rápida, resultando en menores concentraciones que las vacas multíparas T0 entre 18 a 21 DIM (Fig. 8B). En la primera semana después del cambio de manejo (día 25), las vacas primíparas T21 presentaron mayores concentraciones de NEFA que las vacas primíparas T0, sin observarse diferencias posteriormente. En las vacas multíparas, no se detectaron diferencias en las concentraciones de NEFA entre los tratamientos después del cambio de manejo. Sin embargo, el perfil de NEFA de las vacas multíparas T21 presentó un aumento significativo en las concentraciones entre los días 25 y 30, respecto a sus propios niveles preparto, sin observarse diferencias posteriormente. Las concentraciones de BHB también presentaron efecto significativo de la triple interacción de tratamiento, paridad y DIM (P = 0,0201). No se observaron diferencias en las vacas primíparas entre tratamientos, mientras que, tras el cambio al pastoreo, las vacas multíparas T21 presentaron mayores concentraciones de BHB respecto a multíparas T0 (Fig. 8D).

Las concentraciones de insulina fueron mayores en las vacas T21 en comparación con las del grupo T0 ($P < 0{,}0001$), y en las vacas primíparas en comparación con las multíparas ($P = 0{,}0005$). Las concentraciones de insulina también se vieron afectadas por la interacción entre tratamiento, paridad y DIM ($P < 0{,}0001$). Mientras que las vacas primíparas T21 presentaron mayores concentraciones de insulina durante los primeros 21 DIM en comparación con las vacas primíparas T0, con valores similares posteriormente (Fig. 8E), las vacas multíparas T21 mostraron mayores concentraciones de insulina a los 3 y 9 DIM, pero alcanzaron niveles comparables a los de las vacas multíparas T0 posteriormente (Fig. 8F). Esta interacción también reveló diferencias entre paridades dentro del tratamiento, ya que las vacas primíparas en T21 presentaron concentraciones de insulina consistentemente mayores que las vacas multíparas T21 durante la mayoría de los días. Por el contrario, no se observaron diferencias significativas entre paridades en el grupo T0 a lo largo de los días. Las concentraciones de IGF-1 fueron mayores en las vacas T21 que en las T0 ($P = 0{,}0011$), y en las primíparas que en las multíparas ($P < 0{,}0001$). La concentración de IGF-1 se vio afectada por la interacción entre tratamiento, paridad y DIM ($P < 0{,}0001$). Tanto las vacas primíparas (Fig. 8G) como las multíparas (Fig. 8H) T21 presentaron mayores concentraciones de IGF-1 entre los 3 y los 21 DIM en comparación con sus contrapartes en T0, sin diferencias posteriormente. En ambos tratamientos, las vacas primíparas mostraron concentraciones de IGF-1 consistentemente mayores que las multíparas a lo largo del tiempo.

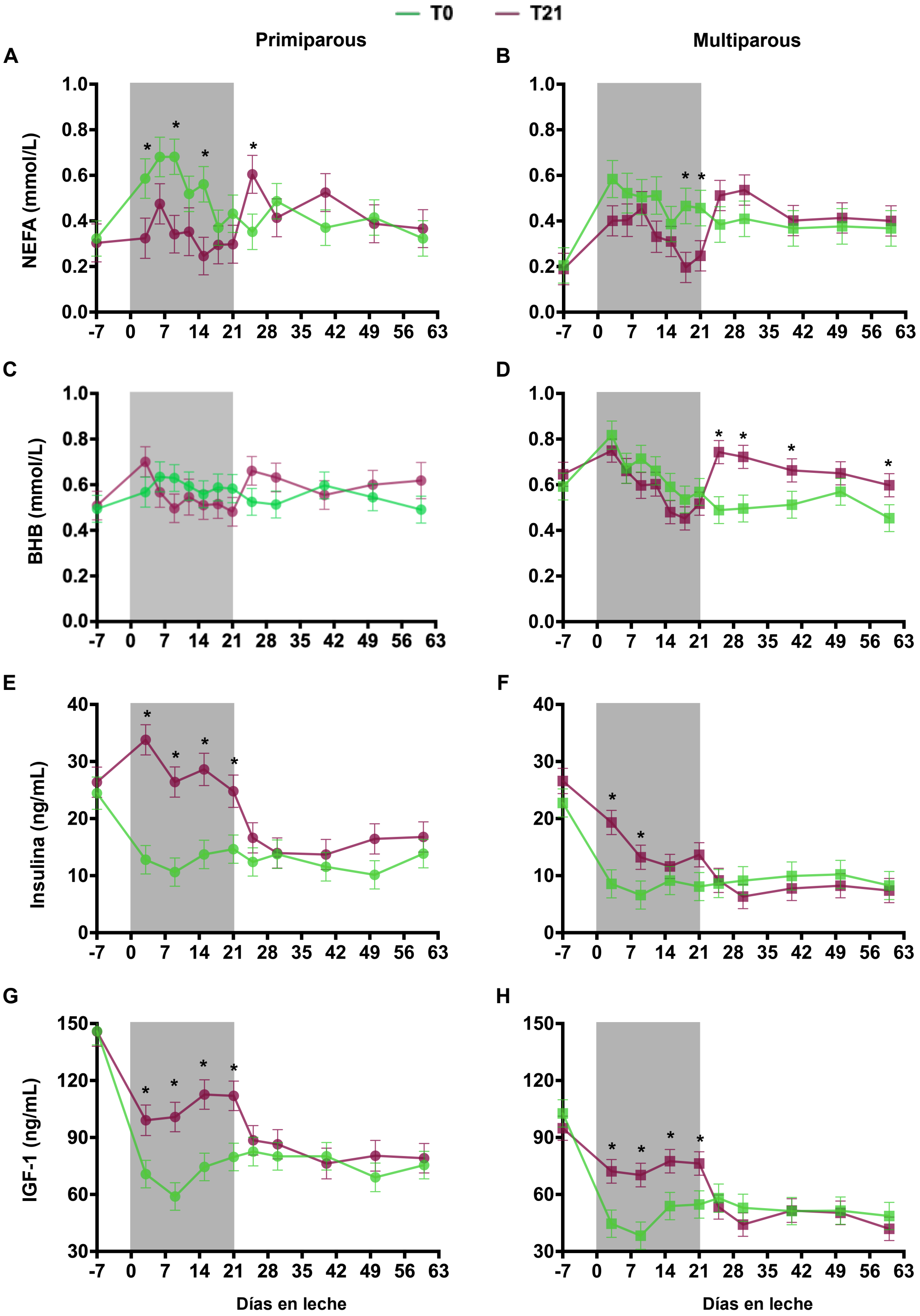

Figura 8. Concentraciones séricas de ácidos grasos no esterificados (NEFA), β-hidroxibutirato (BHB), insulina y factor de crecimiento similar a la insulina -1 (IGF-1), de vacas primíparas (izquierda) y multíparas (derecha) en los tratamientos de pastoreo y suplementación desde el parto (T0) y en estabulación estratégica con TMR *ad libitum* por los primeros 21 días en leche, seguido por pastoreo y suplementación (T21), hasta los 63 días en leche.

El análisis multivariado de metabolómica sérica mostró una clara separación entre los tratamientos en vacas primíparas a los 21 DIM. El modelo OPLS-DA (Fig. 9) presentó una alta capacidad discriminante con un AUC de 0,96 en el análisis ROC y fue estadísticamente significativo según el test de permutaciones. Los metabolitos que más contribuyeron a la separación entre tratamientos fueron glucosa y valina, con concentraciones más altas en el grupo T21, mientras que urea, acetoacetato, BHB, lactato, creatinina, betaína, dimetilglicina, colina, entre otros, se encontraron en mayor concentración en T0 (Fig. 9B). En contraste, no se observaron separaciones claras entre tratamientos en vacas multíparas a los 21 DIM ni en ninguna de las categorías evaluadas a los 60 DIM. Los modelos correspondientes presentaron menor capacidad discriminante (AUC < 0.8) y no superaron la validación por el test de permutaciones.

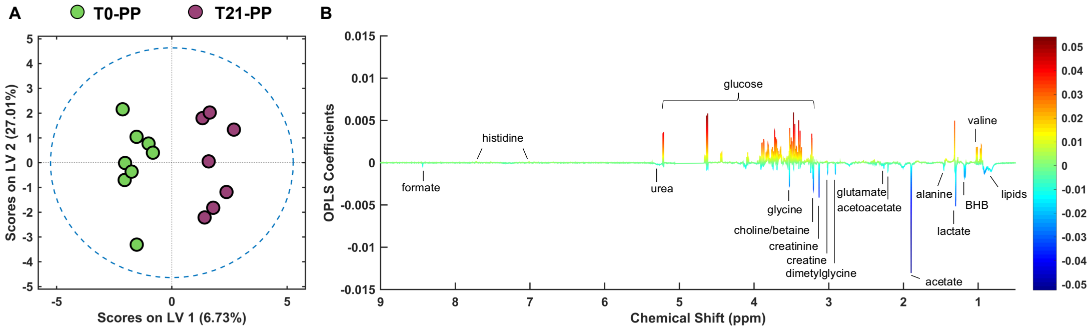

Figura 9. Gráficos de score (A) y de valores de carga (B) obtenidos del análisis discriminante de mínimos cuadrados parciales ortogonales (OPLS-DA), entre vacas primíparas T21 y T0 a los 21 DIM. Los metabolitos que contribuyeron a la separación se encuentran anotados en el gráfico de valores de carga. Los coeficientes R2Y and Q2Y fueron 0,92 and 0,48, respectivamente, y el área bajo la curva (AUC) de la curva ROC fue de 0,96.

Por el análisis univariado, se detectaron efectos significativos de la interacción entre tratamiento y paridad sobre las concentraciones relativas de glucosa ($P < 0,05$), siendo mayores en las vacas primíparas del grupo T21 en comparación con las de T0, mientras que no se observaron diferencias entre tratamientos en vacas multíparas. Además, dentro del grupo T21, las vacas primíparas presentaron mayores concentraciones de glucosa que las multíparas, sin diferencias entre paridades en el grupo T0. Las concentraciones relativas de BHB se vieron afectadas por la interacción entre tratamiento y día, siendo mayores en el grupo T0 que en T21 a los 21 DIM (P = 0.0215), mientras que a los 60 DIM las vacas T21 mostraron concentraciones más elevadas que las T0 (P = 0.0378). Las concentrationes de alantoína tendieron a ser mayores en vacas T0 que T21 (P = 0.0559), principalmente explicado por sus diferencias al día 21 (P = 0.0563), como indicado por la tendencia en la interacción tratamiento por día. Las concentraciones de valina e histidina se vieron afectadas por la interacción entre tratamiento y día, siendo más altas en T21 que en T0 a los 21 DIM, sin diferencias entre tratamientos a los 60 DIM. Las concentraciones de creatinina presentaron una interacción significativa entre tratamiento y día, con mayores niveles en T0 que en T21 a los 21 DIM (P = 0.001), sin diferencias a los 60 DIM. En vacas T21, la creatinina aumentó de 21 a 60 DIM (P = 0.0009), mientras que en T0 se mantuvo sin cambios. Además, hubo una tendencia a la interacción entre tratamiento y paridad: las primíparas T0 presentaron mayores concentraciones que las primíparas T21 (P = 0.0049) y que las multíparas T0 (P = 0.0028). Las concentraciones de formiato fueron mayores en T0 que en T21. Se observó una tendencia a una interacción triple, ya que a los 21 DIM las primíparas T0 presentaron mayores concentraciones que las primíparas T21 (P < 0.0001, Fig. 7P) y también que las multíparas T0 (P = 0.0043), disminuyendo a los 60 DIM respecto de sus niveles a 21 DIM (P < 0.0001).

7.4.Materiales y métodos artículo III

Como parte del mismo experimento descripto para el artículo II, a los 40 y 51 días luego del parto se administró un análogo sintético de prostaglandina (D-cloprostenol, 0.075 mg/mL, Ciclar, Zoovet, Argentina), para la sincronización del estro. Se realizaron extracciones de sangre diariamente durante 10 días a partir de las 24 h (día 1) de la segunda inyección de análogo de prostaglandina para determinación de progesterona sérica. Al día 10 post inyección (aprox. día 7 del ciclo estral y 60 DIM) se extrajeron 2 biopsias endometriales del cuerno uterino ipsilateral al cuerpo lúteo por vía transcervical, que fue inmediatamente almacenada a -196°C y luego a -80°C. Una vez descongeladas, las muestras fueron trituradas utilizando un homogeneizador de tejidos, y tuvieron procesamientos específicos para análisis transcriptómicos y metabolómicos. Para transcriptómica, en el Laboratorio de Análisis Clínicos y Endocrinología y Metabolismo Animal de la Facultad de Veterinaria se extrajo el ARN total con Trizol, acorde a protocolo de fabricante, y se determinó la calidad del ARN mediante nanodrop ND-1000. Se construyeron bibliotecas de secuenciación de extremo pareado (*paired-end*) con un tamaño de inserto de 150 pb, utilizando el kit NEBNext Ultra RNA Library Prep para Illumina (New England Biolabs, Ipswich, MA). La secuenciación se realizó en una plataforma NovaSeq 6000 (Illumina, Sacramento, CA), con una profundidad de lectura promedio de 20 millones de lecturas (*reads*) por muestra. Se evaluó la calidad de los *reads* de la secuenciación mediante FastQC (Andrews, 2010). Cuando necesario, se eliminaron los adaptadores y "reads" de baja calidad, usando los programas scythe (v 0.991, https://github.com/vsbuffalo/scythe) y sickle (v1.33, https://github.com/najoshi/hoz). Luego del procesamiento de cada muestra se realizó un segundo análisis de control de calidad a las muestras procesadas utilizando el programa FastQC. Las muestras con los *reads* limpios se mapearon al genoma de referencia bovino (*Bos taurus*) (ARS-UCD1.2) utilizando el software Hisat2 (v2.1.0) (Kim et al., 2015). Hisat2 se ejecutó con '-k 1', con todos los demás parámetros configurados como predeterminados. Para cada muestra se estimó el número total de *reads* que efectivamente mapearon a cada gen descrito en el archivo de anotación descargado de Ensembl utilizando la función featureCounts de la librería Rsubread (Liao et al., 2014). Los genes diferencialmente expresados (DEGs) se identificaron mediante el paquete DESeq2 (v1.18.1) (Love et al., 2014), considerando aquellos con al menos un *read* en un mínimo de tres muestras.

Se definieron como DEGs aquellos genes con valor de P ajustado ≤ 0.05 (corrección de Benjamini-Hochberg) y $|\log_2 FC| \geq 1$. El área bajo la curva de progesterona y el día del ciclo estral al momento de la biopsia endometrial se incluyeron como covariables. Se realizaron cuatro comparaciones pareadas para evaluar los efectos del tratamiento y la paridad: (i) T0 vs T21 en primíparas, (ii) T0 vs T21 en multíparas, (iii) primíparas vs multíparas en T0, y (iv) primíparas vs multíparas en T21. El análisis de enriquecimiento funcional se realizó con el paquete clusterProfiler en R, utilizando los identificadores de Ensembl convertidos a Entrez mediante org.Bt.eg.db. El análisis de ontología génica (GO, categoría Proceso Biológico) se llevó a cabo con la función enrichGO() y el análisis de vías KEGG con enrichKEGG(), especificando "bta" como organismo. Se aplicó corrección de Benjamini–Hochberg y se consideraron significativos los términos con $p < 0.05$ y $q < 0.1$.

Para el análisis de metabolómica a partir de las muestras endometriales, se agregaron 300 µL de buffer fosfato salino (PBS), se homogeneizó y se centrifugó por 10 minutos a 13800 x g , retirando el sobrenadante que se congeló a -20 °C. Los extractos acuosos de endometrio se dejaron descongelar a temperatura ambiente y se mezclaron alícuotas de 200 µL con 430 µL de solución buffer óxido de deuterio pD 7.4 y se transfirieron a tubos de RMN de 5 mm (NE HL5 7, New Era Enterprises Inc., Vineland, NJ, USA). Los espectros de ^{1}H RMN y el procesamiento de datos se realizó acorde a lo reportado por López Radcenco et al. (2021).

7.5. Principales resultados incluidos en artículo III

La progesterona sérica no tuvo efectos del tratamiento, ni sus interacciones. La interacción entre paridad y día fue significativa, ya que las vacas primíparas mostraron mayores concentraciones de progesterona a partir del día 8, en comparación con las vacas multíparas ($P < 0,0001$; Fig. 10).

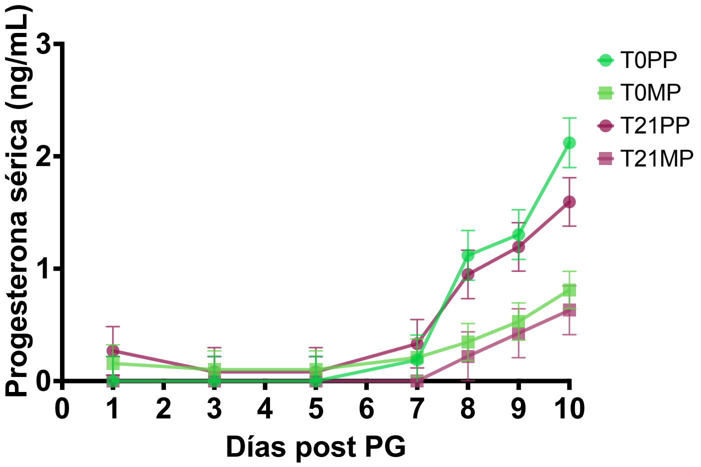

Figura 10. Concentraciones séricas de progesterona luego de la inyección de análogo de prostaglandina (PG, día 0) hasta el día de la biopsia endometrial (día 10), para primíparas (PP, círculos) y multíparas (MP, cuadrados) T0 (verde, vacas en pastoreo con suplementación desde el parto) y T21 (violeta, vacas confinadas con TMR durante los primeros 21 DIM seguido del mismo manejo que las vacas T0).

De los 27.607 genes anotados en el genoma de referencia de *Bos taurus* utilizado, un total de 21.483 genes pasaron los criterios de prefiltrado y se incluyeron en el análisis de expresión diferencial. Sin embargo, dicho análisis reveló poca diferenciación de la transcriptómica endometrial para los distintos contrastes realizados (Fig. 11). En las vacas primíparas, la comparación entre T21 y T0 resultó en la identificación de solamente 15 DEG, mientras que en las vacas multíparas se detectaron 19 DEG para el mismo contraste entre tratamientos. A pesar del uso de criterios estadísticos y biológicos rigurosos (FDR $\leq$ 0.05, $|\log_2 FC| \geq 1$), el número limitado de DEG identificados presentaron en su mayoría niveles bajos de expresión, resultando muchas veces en valores elevados de $\log_2$FC, por lo que se debe tomar estos resultados con cautela. Validaciones de la expresión de algunos de estos genes se realizarán mediante qPCR.

En primíparas, el gen *LPL* (lipoproteína lipasa), una enzima clave responsable de la hidrólisis de triglicéridos circulantes, estuvo altamente expresado en todos los animales, presentando un $\log_2$FC de 1,8 veces superior en primíparas T0 respecto a primíparas T21. Otro gen sobreexpresado en primíparas T0 respecto a T21 fue *RASGEF1A* (*Ras guanine nucleotide exchange factors - domain family member 1A*), que codifica un factor de intercambio de nucleótidos de guanina vinculado a la migración celular y la señalización mediada por Ras, una vía de señalización crucial en las células, principalmente responsable de regular el crecimiento, la proliferación, la diferenciación y la supervivencia celular. Otros genes diferencialmente expresados demostraron muy bajo nivel de expresión, siendo varios de ellos genes no codificantes.

Por otro lado, en multíparas, los DEGs con mayor nivel de expresión fueron *FOS*, *FOSB*, y *IL17RB*, todos sobreexpresados en T0 respecto a T21. El *FOS* se ha asociado con la remodelación y regulación de la respuesta inmune endometrial, además de participar en la regulación endócrina como de la paratiroides (Adhikari et al., 2022). Parte de la misma familia de genes, FOSB, también posee un rol en la proliferación y diferenciación celular, pero está menos caracterizado en el bovino. El gen *IL17RB*, codifica un receptor de citocinas (IL17) que media la respuesta inmunitaria de tipo Th2 (Rickel et al., 2008). Otros DEGs presentaron bajo nivel de expresión, sin embargo se destacan algunos con funciones biológicas relevantes y en algunos casos relacionados a los genes expresados con mayor robustez. Entre ellos, el *CYP19A1*, que codifica la aromatasa, enzima responsable de convertir los andrógenos en estrógenos, también se encontró sobreexpresado en vacas multíparas T0 respecto a T21. Adicionalmente, también con bajo nivel de expresión, pero de potencial relevancia biológica, se encontró una sobreexpresión de ENSBTAG00000039722 en multíparas T0. Este transcripto aún no está caracterizado en bovinos, pero comparte una alta similitud de secuencia (cercano a 100%) al gen que codifica la prostaglandina F sintasa 1 (*PGFS*) en bisonte y otras especies. Otro gen a considerar con cautela por su nivel de expresión, pero sobreexpresado en multíparas T21 y con posible relevancia y coherencia biológica, fue *APOA5*. Este gen codifica la apolipoproteína A5, regulador clave del metabolismo de triglicéridos y sus concentraciones plasmáticas.

Los contrastes entre vacas primíparas y multíparas dentro de los tratamientos revelaron igualmente pocos DEGs, hallándose 19 DEG para las vacas T0 y 12 DEG para las vacas T21. De manera interesante, 4 DEG fueron comunes en ambas comparaciones entre paridades para los dos tratamientos. Entre ellos, el más altamente expresado en ambos contrastes fue *SLC27A6*, el cual estaba sobreexpresado en vacas primíparas respecto a multíparas en ambos tratamientos. Este gen codifica una proteína involucrada en la captación de ácidos grasos de cadena larga en la célula.

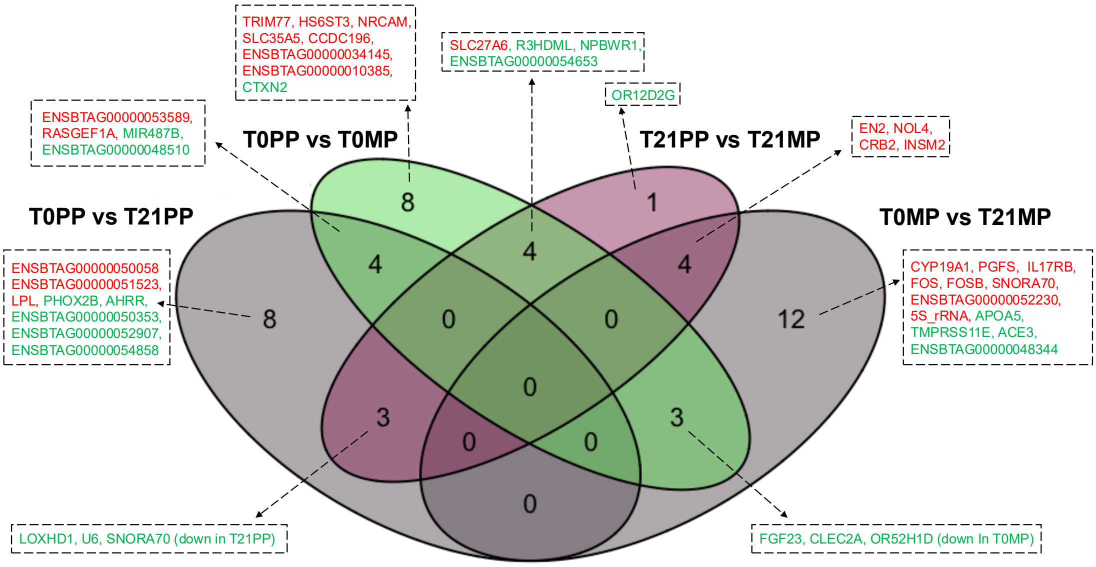

Figura 11. Diagrama de Venn con los genes expresados diferencialmente en los contrastes entre tratamientos (círculos grises) para primíparas (T0PP vs T21PP) y multíparas (T0MP vs T21MP), o entre paridades dentro del tratamiento T0 (verde, T0PP vs T0MP) o T21 (violeta, T21PP vs T21MP), a los 60 días en leche y día 7 del ciclo estral. Los genes sobreexpresados (en rojo) o subexpresados (en verde) se refieren al grupo designado primero en el nombre del contraste, excepto que se aclare lo contrario (genes comunes a más de un contraste identificados en la parte inferior de la figura).

El análisis multivariado de metabolómica endometrial no demostró separaciones claras entre tratamientos en el PCA. Además, el modelo del OPLS-DA presentó una baja capacidad discriminante (AUC = 0,68) y no superó el test de permutaciones. Dado el limitado número de animales por paridad y la exigencia de n mínimo para el test de permutaciones, los análisis multivariados no pudieron ser realizados para comparar los tratamientos separados por paridad. Por este motivo, se complementó con el análisis univariado que permitió realizar las consideraciones para ambos, tratamiento y paridad, siguiendo el mismo modelo utilizado para metabolómica de suero. El análisis univariado reveló diferencias específicas para algunos metabolitos que el enfoque multivariado no logró detectar. Las vacas T0 mostraron concentraciones relativas de lactato más altas que las vacas T21 (P = 0,012). Además, hubo una tendencia a la interacción entre tratamiento y paridad (P = 0,10), ya que los niveles de lactato fueron significativamente más altos en las vacas multíparas T0 en comparación con sus contrapartes en T21 (P = 0,03), mientras que las primíparas no presentaron diferencias entre tratamientos (Fig. 12A). Por el contrario, los niveles de creatina tendieron a ser más altos en las vacas T21 (P = 0,089; Fig. 12B). La concentración de carnitina tendió a ser mayor en vacas T21 (P = 0,071, Fig. 12C) y en vacas multíparas (P = 0,061).

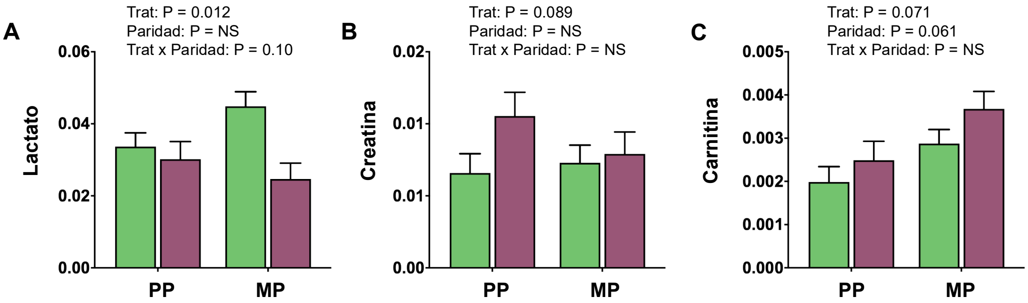

Figura 12. Concentraciones relativas de lactato (A), creatina (B) y carnitina (C) en vacas primíparas (PP) y multíparas (MP) de T0 (verde) y T21 (violeta), determinadas por resonancia magnética nuclear (^{1}H-NMR) a los 60 días en leche y día 7 del ciclo estral.

8. DISCUSIÓN GENERAL

Esta tesis buscó evaluar los efectos del ambiente productivo y del manejo alimenticio sobre la adaptación endócrino-metabólica postparto, y sus consecuencias sobre la performance productiva y variables reproductivas en vacas lecheras en lactancia temprana. Los resultados del experimento I revelaron que bajo condiciones climáticas no extremas, la infraestructura ofrecida en el encierro parcial a cielo abierto no generó grandes diferencias respecto a vacas con encierro parcial en galpón de *compost*. Sin embargo, indicadores metabólicos sugieren que vacas en encierro a cielo abierto adaptaron su metabolismo en respuesta a condiciones ambientales adversas relacionadas a estrés calórico. En cuanto a la comparación del manejo alimenticio, en ambos experimentos se pudo constatar que la vaca multípara logra responder incrementando la producción de leche ante una mayor ingesta de materia seca y energía, pero aun así, mantiene similares niveles de movilización de reservas corporales, al menos en los primeros días postparto, respecto a vacas en pastoreo. Por otro lado, la vaca primípara posee mayor dificultad en la adaptación a este nuevo estado fisiológico de lactación bajo condiciones de pastoreo desde el parto, lo que implica un estatus más catabólico en su organismo. El manejo diferencial de encierro con TMR *ad libitum* favorece esta adaptación, promoviendo un mejor balance energético en la vaca primípara, pero su aplicación solamente durante los primeros 21 días postparto no es suficiente para generar un aumento en producción de leche. Por otra parte, el cambio de manejo al pastoreo luego del periodo de encierro en los primeros 21 días postparto, generó una readaptación metabólica en ambas paridades, que se apoyaron en el uso de sus reservas corporales y ajustaron a la baja la salida energética en leche. Este trabajo presenta limitaciones en cuanto el número de animales para evaluar la variable de reinicio de ciclicidad, pero la evidencia generada en el experimento I, repetida en ambas épocas de parición, parece apuntar que mientras las vacas multíparas están priorizando la producción de leche en respuesta al manejo en confinamiento con TMR *ad libitum*, parecerían relegar temporariamente el reinicio de la actividad ovárica. Por otro lado, este manejo utilizado de manera estratégica en un corto plazo durante los primeros 21 días postparto, no fue suficiente para generar diferencias en términos de probabilidad de reinicio. El efecto residual de este manejo estratégico sobre el ambiente uterino al momento de iniciar los servicios es limitado, y sugiere estar más relacionado al impacto metabólico producido por el cambio de manejo de confinamiento a pastoreo, sobre todo en las vacas multíparas. El resumen esquemático del modelo conceptual propuesto para los hallazgos de la presente tesis y discutidos a lo largo de esta sección están ilustrados en la Figura 13.

Los resultados del experimento I aquí presentados se enfocaron en la evaluación del impacto del ambiente productivo y del manejo alimenticio sobre la adaptación metabólica, la performance productiva y proporción de vacas ciclando en la lactancia temprana. No obstante, es válido resaltar que este trabajo hizo parte de un proyecto mayor que evaluó 4 lactancias completas, 2 para cada época de parto (otoño y primavera), durante 2 años. Por lo tanto, esta discusión se podrá complementar con otros trabajos publicados a partir de este proyecto de carácter multidisciplinar, en el que al día de hoy se ha evaluado su impacto productivo y económico (Méndez et al., 2023; Menegazzi et al., 2025), bienestar y salud animal (Mendina et al., 2023; Pons et al., 2023), perfil de ácidos grasos en leche (Grille et al., 2023), comportamiento ingestivo y aspectos nutricionales en la lactancia temprana (Méndez et al., 2024). De nuestro conocimiento, este fue el primer estudio acerca del impacto de la infraestructura en el encierro (cielo abierto vs *compost barn*) utilizados de manera parcial en combinación con pastoreo. De manera general, no se encontraron mayores diferencias a raíz del tipo de encierro utilizado en confinamiento parcial, excepto que las vacas OD-GRZ con parición de primavera presentaron mayores concentraciones de BHB en etapas más avanzadas de la lactancia (entre 60 y 90 DIM) respecto a CB-GRZ y CB-TMR. Esto fue un hallazgo interesante, dado que el aumento tanto en BHB como en NEFA producto de la lipomovilización se da comúnmente en los primeros 15 DIM (Ingvartsen & Andersen, 2000), como de hecho fue observado en todos los tratamientos en ambas estaciones de parto. Sin embargo, las concentraciones de BHB en vacas OD-GRZ paridas en primavera permanecieron elevadas después de 30 DIM, sin una elevación concomitante de NEFA. Existe evidencia de que las concentraciones de NEFA y BHB no necesariamente aumentan de manera asociada (McCarthy et al., 2015). En este sentido, se debe considerar que además de la oxidación de NEFA, otra fuente de cuerpos cetónicos son los aminoácidos cetogénicos (Sun et al., 2014), principalmente en vacas con baja condición corporal (Pires et al., 2013). Esto concuerda con el presente estudio ya que las vacas de primavera tenían un BCS al parto inferior al recomendado de 3,0-3,25 (Roche et al., 2009). Además, el 42% de las vacas OD-GRZ (datos no presentados) mostraron concentraciones de BHB indicativas de cetosis subclínica (>1,2 mmol/L; McArt et al., 2012) a los 90 DIM, en comparación con el 10% y el 0% en CB-TMR y CB-GRZ respectivamente. Este fue un hallazgo sorprendente, ya que era de esperar que el balance energético negativo ya hubiera cesado en esta fase de la lactación. Sin embargo, dado que la oferta de alimento y el consumo estimado fueron similares entre OD-GRZ y CB-GRZ (ver Tabla 1 artículo 1), así como los niveles de producción de leche, atribuimos estas diferencias al ambiente al que las vacas estuvieron expuestas durante el periodo de encierro, ya que este momento coincidió con periodos de altos valores de índice de temperatura y humedad (ITH>72, ver Figura 1 artículo 1; Polsky & von Keyserlingk, 2017). De hecho, Gantner et al. (2016) encontraron un mayor riesgo de desarrollar cetosis en vacas sometidas a estrés calórico durante la mitad de la lactación, y otros estudios han asociado el estrés calórico con el aumento del catabolismo de las proteínas tisulares (Baumgard & Rhoads, 2013; Gao et al., 2017). Por lo tanto, las instalaciones del encierro a cielo abierto pudieron no ser suficientes para mitigar el calor, como estuvo reflejado en la mayor temperatura subcutánea y adaptación comportamental en las vacas OD-GRZ respecto a CB-GRZ en este experimento (Pons et al. 2023). Estas adaptaciones comportamentales frente a condiciones adversas del ambiente, posiblemente explican la menor eficiencia energética en OD-GRZ comparado a ambos CB-GRZ y CB-TMR, debido a las mayores pérdidas por calor medidas por pulso de oxígeno (Jasinsky et al., 2023). En suma, el análisis integral del experimento permite indicar un efecto detrimental del encierro parcial a cielo abierto en variables que indican bienestar animal, si bien esto no se expresó en diferencias en producción de leche.

El sistema de alimentación fue evaluado en ambos experimentos, tanto de manera estructural -en dos épocas de parición- (Experimento I), como estratégica -tomando en cuenta la paridad: primíparas y multíparas (Experimento II)-. Cuando fue evaluado de manera estructural en vacas multíparas durante los primeros 90 días de lactancia (Experimento I), el confinamiento con TMR *ad libitum* resultó en una producción de leche en CB-TMR que superó en un 29% y un 27% en otoño y primavera, respectivamente, a las vacas CB-GRZ y OD-GRZ. La mayoría de las evaluaciones anteriores con similares sistemas estabulados vs pastoriles con PMR, habían sido realizados en lactancia media, y reportaron una diferencia menor en la producción de leche (5-20%) respecto a la obtenida en el experimento I (Bargo et al., 2002; Mendoza et al., 2016b; Salado et al., 2018, 2020), lo que reafirma la relevancia de la nutrición desde el inicio de la lactancia para maximizar la producción de leche (Kolver & Muller, 1998). Además, en las vacas en pastoreo paridas en primavera se observó una disminución en la producción de leche en el tercer mes de lactancia, lo que posiblemente se explica por una mayor proporción de la pastura en la dieta (~50%, Tabla 1 artículo 1; García & Holmes, 2001) y/o por estrés calórico (Polsky & von Keyserlingk, 2017). Sin embargo, en el experimento II, con pasturas de mejor calidad, las vacas multíparas en pastoreo alcanzaron una producción de alrededor de 40 L/vaca/día, acercándose más al nivel alcanzado por las vacas multíparas en TMR, que en ambos experimentos fue de alrededor de 44 L/vaca día. Aunque en ambos experimentos se observó que la diferencia en producción de leche es visible desde la primera semana postparto, los resultados demuestran que con una nutrición de calidad en sistemas pastoriles, la productividad puede ser igualmente competitiva. Los aspectos de composición de la dieta son importantes, dado que en uno de los antecedentes que evaluaron el uso de TMR estratégico similar al experimento II, la falta de diferencias entre tratamientos en términos productivos fue atribuido a características en la composición de la TMR (menor proporción de almidón y proteína cruda comparado a otros estudios) o desbalances (exceso) en términos de proteína de la pastura (Brady et al., 2021). En el caso de vacas primíparas en el presente estudio, tampoco se encontraron diferencias en producción de leche con un manejo alimenticio diferencial estratégico con TMR *ad libitum* en comparación a vacas primíparas pastoreando desde el parto. Dado que tampoco se encontraron diferencias en vacas primíparas en este ensayo incluyendo partos de otoño (Rivoir et al., 2025), hemos sugerido que el manejo diferencial con TMR por solo 21 días podría no ser suficiente para detectar diferencias. De hecho, en trabajos previos de nuestro grupo, solo se observó una mayor producción de leche en vacas primíparas con TMR, en comparación con sistemas similares basados en pasturas, a partir de 35 DIM (Meikle et al., 2013). De manera general, los manejos alimenticios evaluados presentaron respuestas dependientes de la paridad que reflejaron sus prioridades homeorréticas. Además de la función productiva, la jerarquía de la asignación de recursos energéticos fue también evidente para la función de reinicio de ciclicidad.

La proporción de vacas con cuerpo lúteo no difirió entre los tratamientos pastoriles bajo distintas condiciones ambientales durante el encierro en el experimento I. Sin embargo, ambos fueron superiores a CB-TMR en ambas épocas de parto, en contraposición a la hipótesis de que la TMR *ad libitum* mejoraría el BEN y adelantaría el reinicio de ciclicidad (Butler, 2003; Astessiano et al., 2015). Es posible que las vías metabólicas en CB-TMR estuvieran priorizando el flujo de nutrientes hacia producción de leche en lugar de la función reproductiva, respondiendo a la homeorresis en esta etapa fisiológica (Bauman & Currie, 1980), lo cual es coherente con las pocas diferencias en indicadores endócrino-metabólicos entre tratamientos en vacas multíparas. Además, las vacas multíparas confinadas en este estudio se encontraban fuertemente exigidas en términos productivos, alcanzando mayores niveles de producción respecto a los sistemas pastoriles en el presente trabajo (+28%) en comparación a estudios previos en vacas multíparas de similar genética y alimentación (+12%) en los que no se encontraron diferencias en cuanto a ciclicidad (Astessiano et al., 2015; Fajardo et al., 2015). Esto podría deberse a mejoras en la calidad de la TMR ofrecida que permitió la expresión de su potencial genético, en concordancia con Horan et al. (2004), quienes sostienen que la genética Holstein Americana destina incrementos de energía de la dieta hacia producción de leche, sin una mejora en el rendimiento reproductivo. Esto fue igualmente visualizado en el experimento II, donde a pesar de ser un tratamiento de corta duración, la mayor ingesta de energía en TMR por parte de las vacas multíparas, no significó un adelanto en el reinicio de ciclicidad pero sí en mayor producción de leche. Por otro lado, en vacas primíparas, además de no presentar diferencias entre tratamientos, el reinicio de la ciclicidad en esta paridad estuvo retrasado en comparación al alcanzado por vacas multíparas. Aun cuando las vacas primíparas T21 presentaron gran parte de los indicadores endócrino-metabólicos favorecidos respecto a T0, como también ocurrió con IGF-1 en multíparas confinadas en ambos experimentos, esto no se tradujo en un adelanto del reinicio de ciclicidad en contraposición a la bibliografía (Butler, 2003). En este sentido, De Vries & Veerkamp (2000) encontraron que la magnitud y duración del BEN explicaron solo el 3-4% de la variación en el intervalo hasta la primera ovulación. El balance en sí mismo estaría sujeto a una regulación en el animal, que en el caso de primíparas posiblemente responda a la prioridad para crecimiento, mientras que las multíparas destinan el sobrante energético luego del mantenimiento para la producción de leche en inicio de lactancia (Butler, 2014). Por el contrario, se ha sugerido que vacas en pastoreo se adaptarían al ingreso de nutrientes reduciendo la producción de leche, pero manteniendo niveles aceptables de ciclicidad ovárica

(Butler, 2014). Además, se ha planteado que la alta producción y consumo de materia seca, como la obtenida en sistemas de confinamiento en vacas multíparas, genera un mayor flujo sanguíneo hepático y una depuración acelerada de hormonas esteroideas (ej. estradiol; Sangsritavong et al., 2002). Esto podría afectar el alcance del pico preovulatorio de la hormona luteinizante y la consecuente primera ovulación, conduciendo a una mayor incidencia de quistes foliculares ováricos (Silvia et al., 2002), como se encontró en las vacas CB-TMR de otoño (Adrien et al., 2022). Sin embargo, la proporción de vacas con cuerpo lúteo en otoño fue similar entre los tratamientos a los 60 DIM, coherente con el momento en que las vacas con quistes ováricos reinician su ciclicidad ovárica (Beam y Butler 1998). En un metaanálisis, el nivel de producción también ha sido asociado negativamente con el intervalo entre parto y primer estro, y los autores también proponen una mayor depuración hepática de esteroides sexuales como una posible explicación (Bedere et al., 2018). Sin embargo, en ese estudio el nivel de producción no se asoció con el tiempo al reinicio de ciclicidad ovárica -el cual puede o no ir acompañado de expresión de celo-, pero sí con la condición corporal al parto (Bedere et al., 2018). La función reproductiva de las vacas lecheras responde a interacciones fisiológicas complejas (Berry et al., 2016) y los mecanismos subyacentes en términos de estrategias endócrino-metabólicas adoptadas por los animales bajo los distintos tipos de manejo podrían contribuir a su comprensión.

En ambos experimentos, las vacas multíparas con TMR *ad libitum* presentaron similares indicadores de lipomovilización (NEFA y BHB), respecto a vacas en pastoreo en la lactancia temprana, al menos durante los primeros días en leche. Esto ocurrió a pesar de la mayor densidad energética en la dieta TMR que no se tradujo en un ahorro de reservas corporales en esta categoría, en contraste a hallazgos previos (Astessiano et al., 2015). Esto sugiere que la función primordial de producción de leche en multíparas se sustenta en igual medida por mecanismos homeorréticos de lipovilización independientemente de la alimentación, al compararse con dietas pastoriles de buena calidad, al menos en los primeros días postparto. De los indicadores de balance energético evaluados en el experimento I, únicamente el IGF-1 presentó aumentos en CB-TMR respecto a CB-GRZ y OD-GRZ a partir de los 60 DIM en primavera, lo que es coherente con la mayor disponibilidad de glucosa (o sus precursores) en la TMR (Lucy et al., 2013), respecto a las dietas con inclusión de pastura. Sin embargo, estas diferencias probablemente se detectaron debido a que las concentraciones de IGF-1 en las vacas en pastoreo se mantuvieron bajas, debido al menor consumo alcanzado (Tabla 1 - artículo 1) y la menor densidad energética de la dieta (Obese et al., 2008) dada la mayor participación de pastura en este periodo. Esto fue también observado en el experimento II en el que las concentraciones de IGF-1 fueron superiores en T21 respecto a T0. En este experimento además, la insulina también presentó mayores concentraciones temporarias en la primera semana postparto, sin embargo, a partir de la segunda semana ya no se detectaron diferencias entre tratamientos en vacas multíparas. Esto puede deberse a la mayor capacidad de la glándula mamaria en esta categoría para captar glucosa para la síntesis de lactosa a partir de este momento - observado también en la curva de producción (Fig. 7B; Lucy et al., 2014)-, mediante transportadores independientes de insulina, es decir, GLUT1 (De Koster & Opsomer, 2013). Esta mayor captación probablemente llevó a la reducción de las concentraciones circulantes de glucosa, equiparando la glicemia entre las multíparas de ambos tratamientos como observado en los datos de metabolómica.

Por otro lado, las primíparas T21 siguieron una estrategia distinta, en la que en lugar de convertir el excedente nutricional en leche, el mismo fue volcado a la economía energética corporal, visualizado a través de menores concentraciones de NEFA y mayores concentraciones de glucosa, insulina e IGF-1 durante el periodo de manejo diferencial. Mientras tanto, las vacas primíparas T0 parecieron tener mayor dificultad para adaptarse al pastoreo luego del parto, presentando indicadores de catabolismo lipídico y proteico, que probablemente les permitió equiparar la producción de leche con las primíparas T21. Además de la mayor concentración de NEFA, la metabolómica sérica también reveló mayores concentraciones de alantoína en el grupo T0 respecto a T21 al día 21. La alantoína proviene de la transformación catalizada por uricasa del ácido úrico, un producto final del metabolismo de las purinas (Wang et al., 2016). La misma ha sido asociada con el metabolismo de triglicéridos, estando elevada en vacas con cetosis subclínica (Wang et al., 2016), y vinculada al estrés oxidativo en bovinos (Liao et al., 2018) y humanos (Kand’ár & Žáková, 2008), lo cual es un mecanismo particularmente relevante en esta etapa de postparto inmediato (Sordillo & Mavangira, 2014). Otros hallazgos también sugieren un peor balance proteico en primíparas T0 respecto a primíparas T21, como las menores concentraciones de aminoácidos glucogénicos valina e histidina como reportado por Jorge-Smeding et al.

(2021). Mas aún, sus altos niveles de creatinina estarían indicando un catabolismo de proteínas tisulares (Megahed et al., 2019; Sadri et al., 2023), lo cual también puede ser atribuido a la mayor actividad física debido a la caminata y pastoreo. Los niveles de creatinina fueron igualmente superiores en primíparas que en multíparas T0, pero no se encontraron diferencias entre paridades en T21. Además, vacas primíparas T0 presentaron mayor concentración de dimetilglicina, betaína y formiato, respecto a primíparas T21. La dimetilglicina es generada como un co-producto en la conversión de betaína a metionina. Este proceso libera una unidad de un carbono que puede ser oxidada a formiato, contribuyendo al banco de un carbono en reacciones esenciales como síntesis de nucleótidos y metilación (McFadden et al., 2020). Las vacas lecheras son incapaces de sintetizar metionina en cantidades suficientes para cubrir los requerimientos de síntesis de proteína en leche o para el crecimiento fetal (Arshad & Santos, 2024). Por esta razón, durante el periparto y lactación temprana, las vacas lecheras dependen en gran medida de la conversión de betaína en metionina, así como en el metabolismo del folato para apoyar la metilneogénesis (Xue & Snoswell, 1986), lo que parecería estar aún más acentuado en primíparas T0 con un estatus metabólico más comprometido. El conocimiento del metabolismo de un carbono en vacas lecheras aún es limitado, pero estas diferencias marcadas refuerzan su relevancia no solamente en cuanto a producción de leche, sino también en salud hepática y respuesta inmune, resultando en un área de interés creciente (McFadden et al., 2020). En conjunto, estos datos revelan un estado de mayor déficit energético y proteico en primíparas T0, indicando una mayor dificultad para esta categoría en adaptarse a la lactancia bajo condiciones de pastoreo desde el parto.

Si bien el manejo de estabulación estratégica durante los primeros 21 DIM pudo favorecer en cierta medida la producción de leche en multíparas y el balance energético en primíparas, su implementación podría ser pasible de mejoras. Además de la duración, que pudo no ser suficiente para generar un impacto en producción de leche en primíparas, el cambio de manejo abrupto a partir del día 22 a un turno completo de pastoreo entre ambos ordeñes, generó una respuesta metabólica en ambas paridades para poder adaptarse al nuevo manejo. Aun contando con pasturas de buena calidad y alta oferta de forraje, las vacas de T21 debieron recurrir a sus reservas corporales nuevamente, constatado por el aumento de NEFA y BHB. Las concentraciones de valina e histidina también disminuyeron a los 60 DIM en T21, probablemente por su menor suministro a partir de la dieta pastoril, bien como su mayor utilización como precursor de glucosa o síntesis proteica en leche. También se constató un aumento en las concentraciones de creatinina sérica desde el día 21 al día 60 en vacas T21, sugiriendo una mayor degradación muscular ya sea como fuente de carbono o debido a la mayor actividad física (Megahed et al., 2019; Sadri et al., 2023). Este estatus metabólico adaptativo a la lactancia y a los cambios de manejo, a su vez pudieron impactar el ambiente uterino en términos de transcriptoma y metaboloma.

El bajo nivel de diferenciación en la expresión génica endometrial y metabolómica, es coherente con la forma en que los tratamientos fueron aplicados, dado que al momento de la toma de biopsias uterinas, los animales estaban siendo manejados de la misma manera por aproximadamente 40 días. Además, uno de los mensajeros químicos de mayor impacto a nivel de ambiente uterino, la progesterona, tampoco presentó diferencias entre tratamientos en cuanto a reinicio de ciclicidad o en el perfil circulante luego de la sincronización. Sin embargo, los resultados preliminares de los DEGs detectados, incluyendo los de bajo nivel de expresión, así como los metabolitos, poseen coherencia biológica con el manejo sufrido por los animales, y su adaptación endócrino-metabólica a lo largo del periodo. Estas diferencias fueron más marcadas en multíparas, acorde con el mayor grado de diferencias en indicadores sanguíneos en el periodo posterior al manejo diferencial. En las vacas multíparas T0 hubo un alto nivel de expresión de *IL17RB*, que codifica un receptor de citocinas mediador de la respuesta inmunitaria de tipo Th2 (Rickel et al., 2008), conocido por ejercer importantes funciones inmunitarias necesarias para el establecimiento de la gestación (Talukder et al., 2020). Cuando las interleucinas (IL-17B e IL-17E, también conocida como IL-25), se unen a este receptor , se activa una cascada de señalización intracelular que culmina en la activación del factor de transcripción NF-κB (Wong et al., 2005). Esta vía de señalización desempeña importantes roles para la receptividad endometrial en los primeros días del ciclo estral y preñez (Ross et al., 2010), y la señalización IL17/IL17RB contribuye al desarrollo embrionario (Bie et al., 2017). Estas vías, incluido el gen *IL17RB,* se encontraron enriquecidas en el endometrio de vaquillonas de carne suplementadas con omega-3 (Waters et al., 2014). Este ácido graso poliinsaturado predomina en dietas con pasturas frescas en comparación a dietas con forrajes conservados (NRC, 2001; Krusinski et al., 2022), por lo que la diferencia en la expresión de *IL17RB* podría reflejar un efecto residual de la dieta recibida en los primeros días postparto. También se detectó una sobreexpresión de *FOS* y *FOSB* en vacas multíparas T0 respecto a T21. Se sabe que el *FOS* se encuentra sobreexpresado en el endometrio de vacas gestantes y se ha relacionado con la proliferación y diferenciación celular, la función inmunitaria, la actividad endócrina y la salud endometrial (Adhikari et al., 2022). Aunque con bajo nivel de expresión, *CYP19A1*, gen que codifica la enzima responsable de convertir los andrógenos en estrógenos, también se encontró sobre-expresado en vacas multíparas T0 respecto a T21. Dado que se conoce que el 17 β-estradiol induce la transcripción de *FOS* (Girard et al., 2015), esto estaría en consonancia con la mayor expresión de *FOS* y *FOSB* observada en vacas multíparas T0. Si bien esta enzima es principalmente activa en el ovario, existe evidencia sólida de su expresión en el endometrio humano y de roedores, lo que permite la producción "intrácrina" de estradiol (Gibson et al., 2018), esencial para la formación de un endometrio receptivo capaz de sustentar una gestación viable (Das et al., 2009). Sin embargo, su expresión en el endometrio bovino sigue estando poco caracterizada. Por otro lado, existe evidencia de que el balance energético negativo suprime la expresión ovárica de *CYP19A1* a través del aumento de la inhibina y la disminución de los niveles de adiponectina (Rak et al., 2017). Específicamente, Girard et al. (2015), predijeron la inhibición de la señalización de β-estradiol en vacas con BHB elevado, lo que sería coherente con la baja expresión en multíparas T21 que presentaron mayores niveles de BHB durante todo el periodo post cambio de manejo. A pesar del bajo nivel de expresión del transcripto homólogo a prostaglandina F sintasa 1 (*PGFS*, ENSBTAG00000039722) en multíparas T0, su mayor abundancia respecto a T21 en conjunto con otros genes, podría presentar cierta coherencia biológica. La producción de prostaglandina endometrial en vacas puede ser estimulada por el estradiol (Li et al., 2020), en línea con la sobreexpresión conjunta con aromatasa, y también es regulado por la cascada de señalización vía NF-κB (Waters et al., 2014), presentando coherencia con la alta expresión de *IL17RB*. La prostaglandina desempeña un rol fundamental en los primeros días del ciclo y en la preparación de una posible gestación temprana, regulando la elongación del concepto y los mediadores de las respuestas endometriales a la progesterona y al IFNT en el útero ovino (Dorniak et al., 2011), y se han encontrado concentraciones más elevadas en el lumen uterino en preñez temprana de vaquillonas clasificadas como de alta fertilidad respecto a subfértiles (Moraes et al., 2020). Como mencionamos previamente, si bien la expresión de *APOA5* se debe considerar con cautela por su nivel de expresión, presenta posible relevancia y coherencia biológica. Este gen codifica la apolipoproteína A5, regulador clave del metabolismo de triglicéridos y sus concentraciones plasmáticas (Garelnabi et al., 2013), y se encontró sobre-expresado en multíparas T21. Aunque los mecanismos subyacentes aún no están claros, el aumento de la expresión de *APOA5* promueven la activación de la lipoproteína lipasa (LPL) (Fruchart-Najib et al., 2004; Merkel et al., 2005), lo que podría facilitar la utilización de triglicéridos por los tejidos (Drackley, 1999). Por lo tanto, su mayor expresión en el endometrio de vacas T21 a los 60 DIM podría reflejar una mayor demanda y movilización de energía en ese momento (Khan et al., 2014), en consonancia con las evidencias anteriormente presentadas. De forma coherente con la mayor expresión endometrial de *APOA5* en vacas multíparas T21, la concentración de carnitina también presentó una mayor concentración relativa en este grupo (Fig. 12C). La carnitina desempeña un papel clave en el transporte de ácidos grasos de cadena larga a las mitocondrias para la β-oxidación, asociándose con un aumento de las concentraciones plasmáticas de BHB, sin afectar los niveles circulantes de NEFA (Carlson et al., 2007), coherente con el perfil sérico de multíparas T21. También se ha reportado que la carnitina promueve la gluconeogénesis a partir de aminoácidos como la alanina (Carlson et al., 2007). Es posible que las vacas multíparas T21 se estuvieran apoyando en mayor medida en el uso diversos sustratos como los mencionados, dadas las menores concentraciones relativas de lactato, que podría estar apoyando la formación de glucosa o el suministro de energía en el endometrio (Chen & Dean, 2023), así como un mayor catabolismo de las proteínas corporales que resultó en la mayor concentración de creatina (Sadri et al., 2023). Estos hallazgos podrían indicar una mayor demanda energética en este grupo en comparación con las vacas multíparas T0, dado el impacto del cambio de manejo alimenticio aplicado sobre esta categoría. En conjunto, estas diferencias parecerían indicar un ambiente uterino más favorable para multíparas T0 respecto a multíparas T21. Este experimento no puede discriminar si estas diferencias son consecuencia del estrés metabólico sufrido por las vacas T21 en consecuencia del cambio de manejo, o derivan de efectos residuales de la alimentación diferencial recibida en los primeros días postparto, por lo que se requiere mayor investigación.

En primíparas, las diferencias en términos de expresión génica endometrial fueron menos marcadas, y gran parte de los genes identificados, con bajos niveles de expresión en las muestras, presentan roles de regulación transcripcional o post-transcripcional. Sin embargo, uno de los genes de mayor expresión en las muestras, *LPL*, estuvo sobreexpresado en primíparas T0 en comparación a primíparas T21. El *LPL* codifica la lipoproteína lipasa, una enzima clave responsable de la hidrólisis de triglicéridos circulantes, lo cual podría favorecer la disponibilidad energética para el tejido endometrial. Este gen se encuentra más expresado entre el día 5 y 7 del ciclo estral, respecto a etapas posteriores (13 y 16 d, Forde et al., 2010), y contribuiría a la composición del histotrofo uterino con triacilglicéridos, lo cual puede funcionar como fuente de energía para un posible embrión en desarrollo en esta etapa (Forde et al., 2010; Araújo et al., 2016). Su menor expresión en primíparas T21 abre la interrogante si las mismas pudiesen estar enfrentando un cierto déficit para incorporar estos sustratos al útero, o si estarían utilizando otro tipo de fuentes energéticas al momento de la toma de muestras, lo cual podría ser también coherente con los mayores niveles de creatina encontrados a nivel uterino en T21. A pesar del mayor grado de diferencias endócrino-metabólicas en las vacas primíparas en consecuencia de la estrategia alimenticia durante los primeros 21 DIM, el perfil transcriptómico y metabolómico endometrial al día 60 no presentó mayores diferencias. Esto podría deberse a la dinámica de renovación del epitelio endometrial a lo largo del o los ciclos luego del cambio de manejo a los 22 DIM a partir de cuando sufrieron el mismo manejo. No obstante, se detectaron DEGs relacionados a funciones de regulación transcripcional o post-transcripcional (*U6, MIR487B, AHRR, SNORA70*) con un menor grado de expresión entre las muestras. Así como estos, también se detectaron RNA no codificantes (miRNA o lncRNA) con funciones aún no establecidas en el bovino. Sin embargo, se conoce que este conjunto de genes desempeña roles importantes que pueden estar vinculados a modificaciones epigenéticas en consecuencia de la dieta recibida por la hembra con el potencial de afectar el desarrollo embrionario (Peral-Sanchez et al., 2022). Dados los hallazgos previamente descriptos en metabolómica sérica en primíparas T0 (betaína, dimetilglicina, formiato), es posible que la deficiencia nutricional acentuada en esta categoría en crecimiento en el postparto temprano, pudiera tener repercusiones sobre la metilneogénesis (Custodio et al., 2017; McFadden et al., 2020; Arshad & Santos, 2024) y los procesos de metilación en útero, con consecuencias sobre el éxito reproductivo (Walker & Mitchell, 2013). Mayor investigación en este campo es necesaria para comprender la interrelación entre estos mecanismos.

La comparación entre vacas primíparas y multíparas dentro de los tratamientos también reveló pocos DEGs. Entre los DEGs comunes en ambos contrastes entre paridades, *SLC27A6*, se encontraba sobreexpresado en vacas primíparas respecto a multíparas en ambos tratamientos. Este gen codifica una proteína involucrada en la captación de ácidos grasos de cadena larga en la célula y su expresión en endometrio se asocia positivamente con las concentraciones de progesterona (Forde et al., 2012), lo que concuerda con las diferencias en la concentración de esta hormona entre vacas primíparas y multíparas en el presente estudio. Forde et al. (2009) atribuyeron a *SLC27A6* la capacidad de enriquecer el histotrofo con ácidos grasos para sostener las etapas tempranas del desarrollo embrionario. Sin embargo, Moraes et al. (2018) encontraron una sobreexpresión endometrial de *SLC27A6* en vaquillonas infértiles, en comparación con vaquillonas subfértiles o fértiles no gestantes, lo que podría indicar un mayor compromiso de las vacas primíparas para sostener una gestación en este momento de toma de muestras.

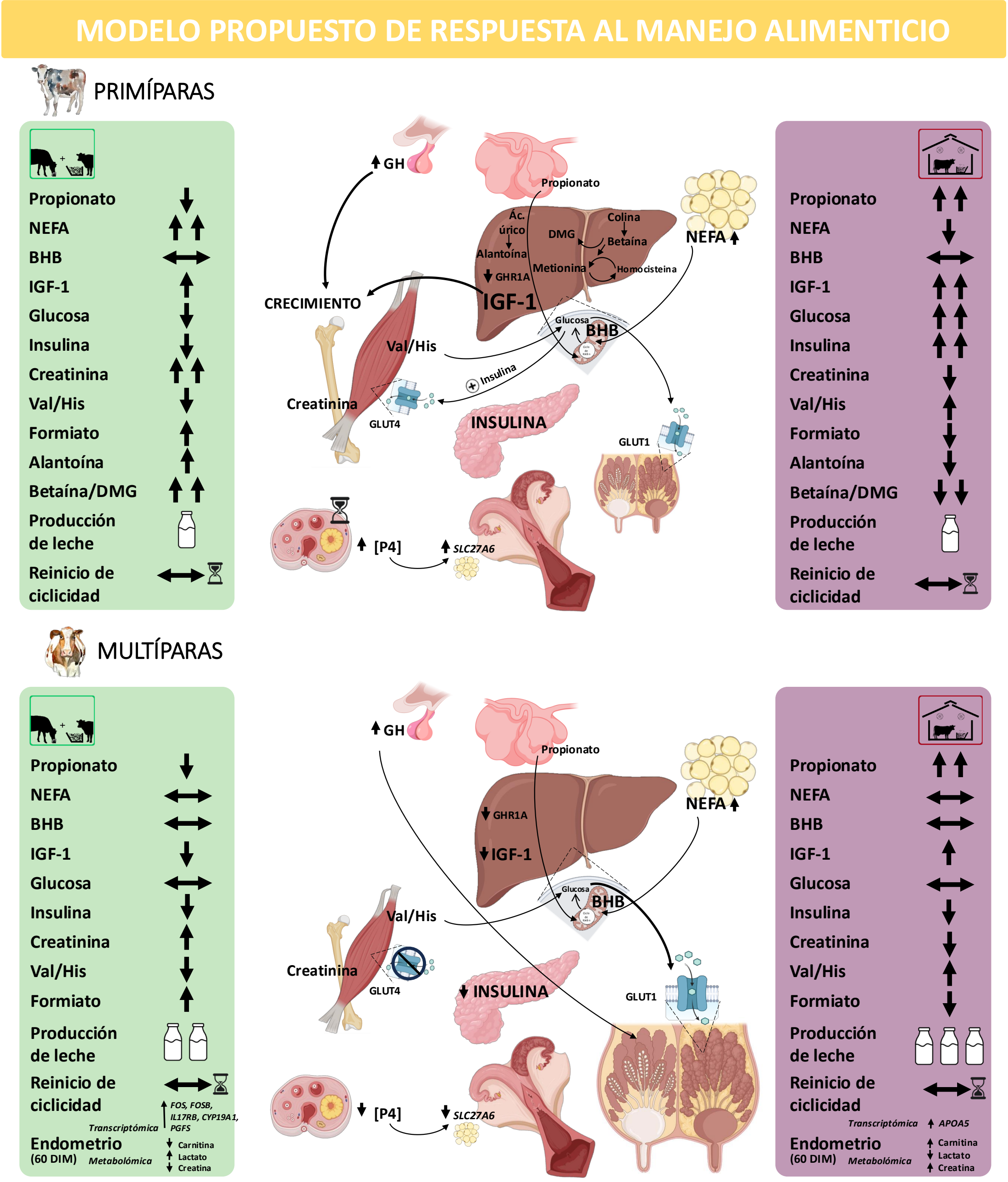

Figura 13. Representación esquemática del modelo conceptual propuesto en base a los hallazgos de la presente tesis, en términos de respuestas endócrino-metabólicas, productivas, y del tracto reproductivo en vacas primíparas (cuadrante superior) y multíparas (cuadrante inferior) de animales en sistemas de base pastoril con suplementación (izquierda) o en confinamiento con TMR *ad libitum* (derecha).

9. CONCLUSIONES

Los sistemas pastoriles con encierro parcial a cielo abierto bajo las condiciones experimentales de esta tesis no presentaron diferencias en la performance productiva e indicadores endócrino-metabólicos respecto a vacas con encierro parcial en galpón de *compost*, a excepción de las concentraciones de BHB. Las altas concentraciones de BHB compatibles con cetosis subclínica entre 60 y 90 DIM en vacas en encierro a cielo abierto indican un esfuerzo metabólico ante condiciones ambientales adversas relacionadas a estrés calórico. En cuanto a la comparación del manejo alimenticio, en ambos experimentos se pudo constatar que la vaca multípara logra responder incrementando la producción de leche ante una mayor ingesta de materia seca y energía en sistemas estabulados con TMR, pero aun así, mantiene similares niveles de movilización de reservas corporales, respecto a vacas en pastoreo y suplementación con PMR desde el parto. Por otro lado, el manejo diferencial de encierro con TMR *ad libitum* por 21 días favorece la adaptación metabólica a la lactancia en la vaca primípara, pero no fue suficiente para generar un aumento en la producción de leche. El cambio abrupto de manejo hacia pastoreo luego del periodo de encierro en los primeros 21 DIM, generó una readaptación metabólica en ambas paridades, que se apoyaron en el uso de sus reservas corporales y ajustaron a la baja la salida energética en leche. La evidencia obtenida en el experimento I, repetida en ambas épocas de parición, sostiene que las vacas multíparas destinan el mayor flujo de nutrientes obtenido de la TMR hacia el aumento de la producción de leche, relegando temporariamente el reinicio de la actividad ovárica. Por otra parte, la estabulación estratégica por solo 21 días, no permitió adelantar el tiempo al reinicio de ciclicidad postparto. Sin embargo, la evidencia preliminar indica que existe un efecto residual de este manejo estratégico sobre algunas variables del ambiente uterino (metabolómica y transcriptómica) al momento de iniciar los servicios (60 DIM) lo cual es relevante ya que las vacas estuvieron 40 días bajo el mismo manejo. Los hallazgos sostienen que los efectos de la alimentación impactan a diversos niveles en el metabolismo, producción de leche y eje reproductivo, y éstos interaccionan entre sí, por lo que el enfoque holístico es el más apropiado para la comprensión de los procesos biológicos.

10. CONSIDERACIONES E IMPLICANCIAS

El estudio de los sistemas de producción animal es complejo y multifactorial, e imposible de abordar de forma integral en una sola tesis, por lo que las conclusiones aquí presentadas deberán tomarse con ciertas consideraciones. A pesar de las pocas diferencias en la comparación de la **infraestructura contrastante utilizada en los encierros complementarios a los sistemas de base pastoril**, sobre las variables analizadas en este estudio, se debe destacar que: i) las condiciones climáticas durante la realización de este trabajo fueron relativamente amigables, y ante eventuales olas de calor, los animales demostraron ser afectados, dada la falta de recursos suficientes para mitigar la exposición al ambiente en los encierros a cielo abierto. En un contexto de cambio climático y aumento de la frecuencia de eventos extremos, las consideraciones hacia el bienestar animal deberán incrementarse de manera a permitir una producción de leche energéticamente eficiente, como también demostrado en estudios complementarios que fueron parte de este proyecto; ii) las condiciones en el encierro a cielo abierto brindadas en este estudio se caracterizaron por proporcionar una superficie amplia con posibilidad de intercalar las áreas de uso, así como construcción de lomas elevadas para contar con un suelo sin exceso de barro y que permitiera el descanso de los animales. Además, se proporcionó un área de sombra y bebederos con dispensador automático que permitían la llegada de agua fresca en circulación, y adecuado frente de comedero. Estas condiciones no siempre son encontradas a nivel comercial, por lo que los resultados obtenidos en esta tesis pueden no ser extrapolables a todos los sistemas con encierro a cielo abierto; iii) los encierros a cielo abierto, como el evaluado en este trabajo, poseen el riesgo de contaminación de suelo y cursos de agua, dada la falta de un sistema que permita canalizar y tratar los efluentes depositados en el mismo. Ante una exigencia creciente del mercado en cuanto al impacto ambiental de los sistemas productivos, estos aspectos deberán ser contemplados por los sistemas comerciales si queremos seguir exportando ¾ de la producción nacional de leche.

En cuanto al impacto de los **sistemas de alimentación**, como es bien documentado, los sistemas confinados con TMR logran un aumento productivo en vacas Holstein multíparas con alto potencial para producción de leche. No obstante, esto no necesariamente significará una mejora en el balance energético durante los primeros días postparto cuando comparados a sistemas pastoriles con suplementación con PMR bien manejados, dado que el excedente diferencial de energía se canaliza a mayor producción de leche. Sin embargo, es posible que en estadíos más avanzados de la lactancia, la facilidad para recuperación de condición corporal sea más acelerada en confinamiento, como constatado a lo largo de la lactancia completa en este proyecto (Menegazzi et al., 2025), lo cual debe igualmente ser considerado con atención para evitar el exceso de condición corporal al secado, predisponente de problemas de salud y reproductivos (Fricke et al., 2023). Posiblemente dada la falta de diferencias en indicadores endócrino-metabólicos y a la priorización de nutrientes para producción de leche, el reinicio de ciclicidad postparto puede no ser distinto, o ser incluso más retrasado en sistemas confinados comparado a sistemas de base pastoril bien manejados. El impacto que este factor, así como las propiedades intrínsecas de la dieta en cada sistema posee sobre la performance reproductiva, deberá ser investigado en mayor profundidad.

Los **manejos alimenticios estratégicos de corto plazo** como el realizado en el experimento II, o el manejo diferencial de lotes dentro del tambo, han tenido un interés creciente a nivel comercial en nuestra región. A pesar de la falta de impacto a nivel productivo en primíparas en el presente trabajo, se debe considerar una posible extensión del tiempo de aplicación, así como el posible impacto en términos de desarrollo corporal que este manejo podría proporcionar en esta categoría. De todas maneras, en ambas categorías, el grado de respuesta es dependiente de la calidad de la TMR proporcionada, para lo que sabemos que la gran variación en la composición química de las reservas utilizadas representa un desafío para los productores, pudiendo afectar la viabilidad económica de su implementación.

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12. ANEXOS

Artículo I

Metabolic adaptation to lactation of dairy cows in two contrasting facilities involving partial confinement plus grazing or total confinement. **Mendina GR**, Damián P, Meikle A, Mendez, MN, Chilibroste P, Adrien L. Animal Production Science 64 (2024) AN23383. doi: 10.1071/AN23383.

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ANIMAL PRODUCTION SCIENCE

Metabolic adaptation to lactation of dairy cows in two contrasting facilities involving partial confinement plus grazing or total confinement

G. R. Mendina[A,*], J. P. Damián[B], A. Meikle[C], M. N. Méndez[A], P. Chilibroste[D] and M. L. Adrien[A]

For full list of author affiliations and declarations see end of paper

***Correspondence to:**
G. R. Mendina
Departamento de Ciencias Veterinarias y Agrarias, Facultad de Veterinaria, CENUR Litoral Norte, Universidad de la República, Ruta 3 km 363, Paysandú 60000, Uruguay
Email: g.rmendina@gmail.com

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ABSTRACT

Context. The increasing intensification of pasture-based systems has subjected the cows to different productive environments, which could affect physiological responses and, consequently, their productive performance. **Aims.** The aim was to determine the effect of contrasting housing facilities (outdoor soil-bedded vs compost-bedded pack barn) used in partial confinement in pasture-based systems, on the metabolic adaptation during early lactation and its impact on productive and reproductive performance of autumn- and spring-calving dairy cows, having as a positive control a zero-grazing confined system in compost barn. **Methods.** Multiparous Holstein dairy cows that calved in autumn ($n = 36$) and spring ($n = 48$) were distributed in the following three treatments from calving to 90 days in milk (DIM): outdoor soil-bedded–grazing (OD-GRZ), compost barn–grazing (CB-GRZ), compost barn–total mixed ration (CB-TMR). Milk production, total milksolids (TMS), body condition score, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), cholesterol, insulin, insulin-like growth factor-1 (IGF-1), and the proportion of cows with corpus luteum were determined in repeated measurements. **Key results.** In both calving seasons, milk production and TMS yields did not differ between OD-GRZ and CB-GRZ, but were greater in CB-TMR ($P < 0.0001$). In autumn, NEFA and BHB were not affected by treatments, but cholesterol increased faster in CB-TMR ($P = 0.0500$). In spring, NEFA and cholesterol concentrations were not affected by treatment, but BHB remained greater in OD-GRZ until 90 DIM than in the other treatments. IGF-1 and insulin did not differ between treatments in autumn, but in spring, IGF-1 was greater in CB-TMR ($P < 0.0001$). The proportion of cows with corpus luteum was not different between pasture-based treatments, but both were greater than CB-TMR up to 40 DIM in autumn ($P = 0.0489$) and during the entire study in spring CB-TMR ($P = 0.0285$). **Conclusions.** Although no differences were found among housing facilities in partial confinement, except that in spring OD-GRZ cows had greater BHB concentrations, confined cows prioritised milk production instead of reproductive function, despite presenting better energy indicators than did pasture-based cows. **Implications.** Outdoor housing combined with grazing can increase the risk of greater BHB concentrations, indicative of subclinical ketosis, under heat-stress conditions, when compared with indoor housing. Confined cows increased milk production but had a delay in the resumption of ovarian cyclicity, even having a better energy status than pasture-based cows.

Keywords: compost-bedded pack barn, early lactation, farming systems, metabolism, outdoor soil-bedded, pasture-based, reproduction, TMR.

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Artículo II

Feeding strategies during fresh cow period in pasture-based dairy systems: metabolic adaptation to lactation and resumption of ovarian cyclicity in primiparous and multiparous cows. **Mendina GR***,* Adrien ML, López-Radcenco A, Moyna G, Rivoir C, Chilibroste P, Meikle A. En revisión en Journal of Dairy Science.

Interpretive Summary: Strategic TMR-confinement during the first 21 days postpartum in pasture-based dairy systems elicits parity-dependent responses. This strategy improved energy balance and increased milk yield by 12% in multiparous cows, with no production benefits in primiparous cows compared to those grazing with supplementation from calving. Primiparous cows grazing from calving experienced a greater metabolic challenge, with increased lipid and protein mobilization, whereas those fed TMR showed a better energy status. After transitioning to grazing, both parities underwent further metabolic adjustments and reduced milk production. The feeding strategy did not shorten the postpartum anestrus period.

Running head: Metabolic adaptation to fresh cow feeding management

Feeding strategies during fresh cow period in pasture-based dairy systems: metabolic adaptation to lactation and resumption of ovarian cyclicity in primiparous and multiparous cows

Graciana R. Mendina [a], María de Lourdes Adrien [a], Catalina Rivoir [c], Andrés López-Radcenco [b], Guillermo Moyna [b], Pablo Chilibroste [c], and Ana Meikle [d]

[a] *Departamento de Ciencias Veterinarias y Agrarias, Facultad de Veterinaria, CENUR Litoral Norte, Universidad de la República, Ruta 3 km 363, Paysandú 60000, Uruguay*

[b] *Departamento de Química del Litoral, CENUR Litoral Norte, Universidad de la República, Ruta 3 km 363, Paysandú, 60000 Uruguay.*

[c] *Departamento de Producción Animal y Pasturas, Facultad de Agronomía, Universidad de la República, Ruta 3 km 363, Paysandú 60000, Uruguay.*

[d] *Laboratorio de Endocrinología y Metabolismo Animal, Facultad de Veterinaria, Universidad de la República, Ruta 8 Km 18, Montevideo 13000, Uruguay*

Corresponding author: Graciana R. Mendina. E-mail: g.rmendina@gmail.com

Abstract

The study aimed to determine whether confinement with total mixed ration (TMR) during the first 21 days in milk (DIM), followed by grazing supplemented with partial mixed ration (PMR), alleviates negative energy balance, enhancing productive performance and accelerating the resumption of ovarian cyclicity in primiparous and multiparous dairy cows, relative to a control group managed on grazing supplemented with PMR after calving. Following calving, 16 primiparous and 24 multiparous Holstein dairy cows were blocked and randomly distributed in two treatments: one included grazing plus supplementation with PMR after calving (T0), while the other one involved confinement with TMR ad libitum during the first 21 DIM and the same feeding management of T0 from day 22 onwards until 60 DIM (T21). Primiparous cows showed no significant differences between treatments in milk production. However, T21 primiparous cows displayed lower non-esterified fatty acids (NEFA), greater glucose, and greater insulin and IGF-1 concentrations compared to T0 primiparous cows during the 21 DIM of the feeding management. In contrast, multiparous T21 cows achieved greater milk production during the first 21 DIM, with no differences in NEFA and BHB levels but greater insulin and IGF-I concentrations than multiparous T0 cows. Both parity groups in T21 underwent an extra metabolic adaptation following the management change at 22 DIM, increasing NEFA and BHB concentrations, and decreasing milk production during this period. Despite the improved endocrine-metabolic profile observed in T21 during the first days postpartum, no differences were found in the resumption of ovarian cyclicity which was shorter in multiparous than primiparous cows. Untargeted metabolomics supported evidence that primiparous cows grazing from calving had greater lipid and muscle mobilization than other groups, reflected by lower glucose and greater creatinine, dimethylglycine, and formate. Strategic feeding management during the fresh cow period affects the metabolic adaptation to lactation, but milk production responses were observed only in multiparous cows, reflecting parity-specific homeorhetic priorities.

Keywords: Early lactation, Transition period, Metabolism, Parity, Metabolomics.

Author-defined abbreviations: NEB = negative energy balance; NEFA = non-esterified fatty acids; PMR = partial mixed ration; T0 = Cows grazing in one session and supplemented with PMR from calving to 60 DIM; T21 = cows fed a TMR ad libitum in confinement from calving until 21 DIM, and managed as T0 cows from 22 to 60 DIM.

Introduction

The transition period, defined as the 21 days before and after calving (Grummer, 1995), represents a critical phase during which dairy cows undergo profound endocrine-metabolic shifts and are subjected to new management conditions. Particularly, the early postpartum period (Cardoso et al., 2020) is characterized by a rapid increase in metabolic and nutrient demands essential for the onset of lactation (Bell, 1995). Successful adaptation to the lactating state, along with the early resumption of ovarian cyclicity to facilitate conception, ultimately determines cow's longevity in the herd and farm profitability (Drackley, 1999). The well-documented negative energy balance (NEB) during this period, marked by elevated concentrations of non-esterified fatty acids (NEFA) and BHB along with reduced insulin and IGF-1, has been associated with compromised productive and reproductive outcomes (Patton et al., 2007; Butler, 2014). This endocrine-metabolic response and NEB are primarily genetically regulated to support milk production via homeorhetic pathways (Bauman and Currie, 1980; Friggens et al., 2004), yet physiological homeostasis is also responsive to nutritional management (Friggens et al., 2004; Roche et al., 2009). Maximizing dry matter and energy intake in the early postpartum could mitigate the severity and duration of NEB, and has been extensively studied to enhance both productivity and reproductive performance (Cardoso et al., 2020).

Pasture-based systems offer advantages from an economic perspective (White et al., 2002) and are well-received by consumers (Cardoso et al., 2019); however, NEB is generally more pronounced compared to confinement systems (Kolver and Muller, 1998). This is due to the morphologic and chemical characteristics of pasture as well as increased energy expenditure related to walking and grazing activities (Kolver and Muller, 1998; Talmón et al., 2025). Various strategies, including increased supplementation and combinations of grazing with partial mixed ration (PMR), have been evaluated (Bargo et al., 2002; Meikle et al., 2013; Fajardo et al., 2015; Mendoza et al., 2016; Méndez et al., 2023). Nonetheless, even when grazing accounted for only one-third of the diet alongside PMR, energy status indicators in early lactation were still poorer compared to fully confined cows (Astessiano et al., 2015). Thus, a short-term confinement during the initial critical postpartum days may serve as a tool to enhance nutrient intake and reduce energy expenditure, though research on this approach remains scarce and inconclusive (Al Ibrahim et al., 2013; Brady et al., 2021). Feeding strategies based on total mixed ration (TMR) during the first 21 (Al Ibrahim et al., 2013) or 30 days in milk (DIM; Brady et al., 2021) did not increase milk production compared to cows grazing after calving, but some carry-over effect on greater milk yield was suggested (Brady et al., 2021). Although this management resulted in some improved endocrine-metabolic indicators, no meaningful differences in the resumption of ovarian cyclicity were found (Al Ibrahim et al., 2013; Brady et al., 2021).

The adaptation to lactation and the response to early postpartum feeding depend on parity. Multiparous dairy cows prioritize milk production while primiparous cows calving for the first time at young age (e.g., 24 months of age) must allocate nutrients for calf growth and milk production while still completing their growth (Wathes et al., 2007; Meikle et al. 2018). Moreover, primiparous cows, which constitute about 30% of the herd, face additional challenges in adapting to first lactation due to interactions with the remaining two-thirds multiparous cows (Wathes et al., 2006; Proudfoot and Huzzey, 2022). Reports on the energy status of primiparous versus multiparous dairy cows are mixed, showing either better (Wathes et al., 2006; Morales Piñeyrúa et al., 2018) or worse outcomes (Meikle et al., 2004; Berry et al., 2006). Such discrepancies can be attributed to factors like feeding management, age at first calving, and body condition score, among others. In a recent study (Rivoir et al., 2025), we have shown that the response to contrasting TMR vs grazing plus PMR feeding systems at the onset of lactation (21 DIM) was dependent on parity. Indeed, while multiparous TMR cows increased their milk production when compared to PMR+grazing cows, no differences were observed in primiparous cows (Rivoir et al., 2025). However, no studies have compared the endocrine-metabolic adaptation to lactation in primiparous and multiparous cows under contrasting TMR vs grazing plus PMR feeding systems at the onset of lactation (21 DIM), and their transitioning from the indoor TMR-based diet to that pasture-based system.

This study hypothesizes that TMR confinement during the first 21 DIM modifies the metabolic adaptation to lactation and, in consequence, the productive and reproductive performance, compared to cows managed on grazing with PMR supplementation after calving, and that the response to the management is dependent on parity. Thus, this study aimed to determine whether TMR-confinement during the first 21 DIM, followed by grazing with PMR supplementation, endocrine-metabolic indicators, and their association with milk production and resumption of ovarian cyclicity in primiparous and multiparous dairy cows, relative to a control group managed on grazing plus PMR supplementation after calving.

Material and methods

This study was performed at the Estación Experimental Mario A. Cassinoni, Facultad de Agronomía, Universidad de la República, Paysandú, Uruguay (32° 23'07.6 "S 58° 03'17.9" W). The protocol was approved by the ethics committee of the Universidad de la República (Comité de Ética en el Uso de Animales de Experimentación, CEUA-CHEA ID 1344, exp. 020300-501632-21).

Animals and treatments

The study was performed with 16 primiparous and 24 multiparous Holstein dairy cows that calved in winter. Sample size calculations were performed through Proc POWER (SAS OnDemand for Academics, v. 3.1.0, SAS Institute Inc., Cary, NC, USA), assuming an expected mean difference of 10% for all variables, with a significance level of 0.05 and a power of 0.8, based on previous reports (Kolver and Muller, 1998; Bargo et al., 2002; Vibart et al., 2008; Meikle et al., 2013; Mendina et al., 2024). Sample size results ranged from 8 to 12 cows per treatment, depending on the variable. Before calving, cows were blocked according to the number of lactations, expected calving date, body weight (BW), and BCS, and then randomly distributed into two treatments which started immediately after calving until 60 DIM. One incorporated grazing plus supplementation with PMR after calving (T0), and the other one involved confinement with TMR ad libitum during the first 21 DIM and the same feeding management as T0 from day 22 onwards (T21). Productive variables and ingestive behavior of the cows used in this experiment have been previously published (Rivoir et al., 2025). All the animals underwent a clinical examination by a veterinarian between 5 and 10 days postpartum to check their health status and ensure their continuity in the experiment. Because of calving complications or serious illnesses (caesarean section, downer cow syndrome, metritis), some animals were removed from the experiment. The final number of animals enrolled in the experiment included 15 primiparous (8 and 7 in the T0 and T21 groups, respectively) and 19 multiparous (8 and 11 in the T0 and T21 groups, respectively). The range of the calving date was of 25 days between first and last calving, the mean number of lactations of multiparous was 2.7 ± 1.1, BW at calving was 566 ± 48 for primiparous and 665 ± 89 kg for multiparous, and BCS at calving was 3.2 ± 0.2 and 3.3 ± 0.2 for primiparous and multiparous, respectively.

All animals had the same prepartum management in separate paddocks according to parity and were fed a TMR with anionic salts. During the postpartum period, primiparous and multiparous cows were managed together within each treatment, reflecting commercial practices. After calving, cows in T0 went out to graze between morning and afternoon milking (7:00 to 14:00 h) and stayed in an outdoor soil-bedded yard where supplementation with PMR was offered, during the rest of the day. The outdoor yard had natural shade and automatic water troughs. Cows in T21 were housed in a compost-bedded pack barn during the first 21 DIM with ad libitum TMR, coming out only for milking. The compost barn consisted of a roofed barn, with ventilation (both natural and with fans) and sprinklers for cooling the animals in the feed alley. The area of compost-bedded pack was 13.5 m²/cow. There was an adjacent feeding area of concrete with 6.75 m²/cow with automatic water troughs and feed bunks with a linear space of 0.77 m/cow. The bedded pack management is described by (Mendina et al., 2024). From 22 DIM onwards, cows of T21 joined T0 treatment and were managed together until 60 DIM.

The amount of TMR offered to T21 was adjusted to obtain an ad libitum consumption (approximately 10% of feed refusal). A PMR was supplied for cows under T0 treatment to complement pasture intake, according to the weekly pasture allowance (assuming

50% of pasture utilization). The daily herbage allowance was at least three times higher than the expected DMI (25 to 30 kg DM/cow/day at ground level). Forage resources were paddocks with a third-year mixed pasture of *Medicago sativa* and *Dactylis glomerata*, a first-year pasture of *Lolium multiflorum, Cichorium intybus,* and *Trifolium pratense,* second-year pasture of *Festuca arundinacea*, and annual grass of *Avena sativa* or *Lolium multiflorum*. Grazing was carried out in a weekly occupation rotating throughout grazing paddocks. Pasture supply was adjusted weekly based on pasture growth rate and herbage condition at the start of grazing (number of expanded leaves for grasses or nodes for lucerne), and the herbage mass in the grazing area. The ingredients used in TMR and PMR are shown in Table A1, and the average chemical composition is shown in Table A2. The characteristics and average composition of the pasture are shown in Table A3.

Routine and sample collection

The cows were milked twice a day at 4:00 and 15:00 h. A complete milking routine was performed every milking, as described by Mendina et al. (2023). Milk production was registered daily by an automatic recording system (GEA Farm Technologies, Inc) from 7 to 60 DIM. Milk samples were collected weekly during both daily milking for determination of fat, protein, and lactose and analyzed using near-infrared spectroscopy (Milkoscan Minor, Foss, Hillerød, Denmark). Total milk solids were calculated as the sum of daily kilograms of fat, protein, and lactose from 7 to 56 DIM. Weekly, from prepartum to 60 DIM, BCS was assessed and recorded by the same observer using a 5-point scale (Edmonson et al., 1989). Blood samples were taken from the coccygeal vein using an evacuated tube system (Vacuette 8 mL Serum Beads Clot Activator, Greiner Bio-One GmbH) from one week before calving until 60 DIM.

Blood samples were centrifuged at 1,680 x g for 10 minutes at room temperature and serum was stored at -20 °C until further analysis. Postpartum measurements of NEFA, BHB, and cholesterol, were carried out two times a week from calving to 30 DIM and then every 10 days until 60 DIM. Insulin and IGF-1 were analyzed once a week until 30 DIM, and then every 10 days until 60 DIM. Serum progesterone (P4) was determined two times per week from 12 DIM until detection of resumption of ovarian cyclicity, defined as the first day in which the concentration of serum P4 was greater than 1ng/mL, remaining high in the following sampling (Adrien et al., 2012). Serum samples from 21 and 60 DIM were collected for metabolomics analysis.

Metabolite and hormone determination

Serum NEFA, BHB, cholesterol, insulin, IGF-1, and progesterone concentrations were determined at the Laboratorio de Endocrinología y Metabolismo Animal, Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay. Metabolites were measured by colorimetric assays on spectrophotometry (BA200, Biosystems S.A, Barcelona, Spain) using commercial kits: NEFA-HR 2 (Fujifilm Wako Pure Chemical Industries Ltd.), BHB, cholesterol (Biosystems S.A, Barcelona, Spain). The controls used were those included in the kit and internal laboratory controls. The interassay and intraassay CV for all determinations was less than 10%. Serum insulin concentrations were determined by solid-phase radioimmunoassay (RIA) in a single assay using the INS-IRMA kit (DIA Source Immune Assays S.A., Belgium). The assay sensitivity was 1.3 µIU/mL, and the intra-assay CV for control 1 (19.4 µIU/mL) was 6.3%, and for control 2 (65.6 µIU/mL) 4.0%. Serum IGF-1 analysis was performed using an automated solid-phase chemiluminescent immunoassay kit in conjunction with an IMMULITE 1000 System (Siemens Healthcare Diagnostics, United Kingdom) calibrated according to the instructions provided by the manufacturer. The interassay CV for control 1 (142 ng/mL) and control 2 (224 ng/mL) were 8.9 and 6.9%, respectively. The limit of detection for IGF-1 analysis was 14.4 ng/mL. Serum progesterone was analyzed by a solid-phase radioimmunoassay (RIA) using a commercial kit (MP Biomedicals, Los Angeles, CA, USA), as reported by Ruprechter et al. (2020). The assay sensitivity was 0.11 ng/mL and the intra- and inter-assay CV for Control 1 (0.5 ng/mL) were 19 and 21%, respectively, and for Control 2 (5 ng/mL) 8.9 and 16.6%, respectively.

Sample preparation and proton nuclear magnetic resonance (^{1}H-NMR) spectral acquisition

Blood serum samples were allowed to thaw at room temperature, and 200 µL of aliquots were mixed with 400 µL of a deuterium oxide buffer solution pD 7.4 and transferred to 5 mm NMR tubes (NE HL5 7, New Era Enterprises Inc., Vineland, NJ, USA) as reported by Dona et al. (2014). Quality control samples were prepared by pooling equal aliquots of all serum samples and were analyzed intermittently throughout the analytical sequence. Their spectra were also acquired, and a principal component analysis (PCA) was performed at the end of the run to verify that QC samples clustered tightly at the center of the dataset, indicating analytical stability (Fig. A1). All NMR spectra were recorded at 25 °C on a Bruker AVANCE III 500 NMR spectrometer operating at ^{1}H and ^{13}C frequencies of 500.13 and 125.76 MHz, respectively (López-Radcenco et al. 2021). 1D ^{1}H free induction decays were zero-filled to 64 K points and apodized with a 0.3 Hz exponential window function before Fourier transformation. ^{1}H NMR spectra were manually phased and baseline corrected using MNova (version 10.0, MestreLab Research, S.L., Santiago de Compostela, Spain) and referenced to the α-glucose resonance at 5.22 ppm present in all serum samples. Manually-selected spectral regions were aligned, and the data was normalized to the total spectral area after excluding the residual water resonance signal (4.60–5.00 ppm). The resulting data matrix was then exported as a text file for multivariate analyses.

Metabolite identification and quantification

Metabolites were identified by comparison of 1H NMR data against spectral repositories, including the Biological Magnetic Resonance Bank (BMRB) (Hoch et al., 2023), the Human Metabolome Database (HMDB) (Wishart et al., 2022), and Chenomx (version 9, Chenomx, Inc., Edmonton, Canada). When required, metabolite identification was confirmed with data from 1D-TOCSY and HSQC spectra. Variations in the levels of unambiguously identified serum metabolites were estimated using relative concentrations. This figure was computed as the ratio between the area from individual metabolite ^{1}H NMR signal and the total area of the spectrum.

Statistical analysis

For milk production, TMS, BCS, metabolites, and hormones, the assumption of normality was determined by the Shapiro-Wilk statistic. All those variables were analyzed using a generalized linear mixed model (GLIMMIX procedure; SAS Studio®), with fixed effects defined as treatment, DIM, parity, and their interactions. When the interaction was not significant, the term was removed from the model, as described by Brady et al. (2021). The block was considered a random effect. The cow was used as the experimental unit. The covariance structure was compound symmetry for milk and total milk solids production, and autoregressive order 1 for BCS, metabolites and hormones, as they better fitted the Akaike information criterion (AIC) value. Variables with a single data point such as delta BCS (ΔBCS) at 21, ΔBCS at nadir, and days at nadir were analyzed using GLIMMIX procedure of SAS (SAS Studio®) as described in Brady et al. (2021). For these analysis, fixed effects were defined as treatment, parity, and the interaction between treatment and parity. The BCS at calving was used as a covariate in all BCS related analyses. Significance was considered with alpha ≤ 0.05, and a tendency between 0.05 and 0.10. Post hoc comparisons were performed with Tukey–Kramer test. The probability of resumption of ovarian cyclicity analysis was carried out using Cox's proportional hazards regression models (PHREG procedure; SAS Studio®), including as fixed effects the treatment and parity. When significant, the outputs are presented as a hazard ratio (HR) set to the reference group, where a HR >1 means that an event occurs sooner, whereas a HR <1 means that an event will occur later (Cox, 1972). Survival curves illustrating the evolution of resumption of ovarian cyclicity per parity and treatment are presented. Multivariate statistical analyses, including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), were carried out with the PLS_Toolbox package (version 8.5, Eigenvector Research Inc., Manson, WA, USA) implemented for MATLAB (revision 2017b, The MathWorks Inc., Natick, MA, USA). For all models, the data was mean-centered and scaled using a Pareto factor (van den Berg et al., 2006). Cross-validation of all OLPS-DA models was achieved using the random subset method, which involved 20 iterations over data split into 8 equally sized parts. Receiver operating characteristic (ROC) curves were plotted, and area under the curve (AUC) values were calculated to ensure the goodness of fit of the resulting models. A permutation test with 50 iterations was also performed to determine the degree of over-fitting and further validate the discriminant analyses (Ni et al., 2008). The results from these validations are provided in Appendix (Figures A2–A3). Metabolomics univariate analyses were performed as described for metabolites.

Results

Milk production, total milk solids, and probability of resumption of ovarian cyclicity

Milk production was affected by parity (Table 1) as multiparous cows produced 36.5% more milk than primiparous cows (40.0 vs 29.3 L/cow/day). The triple interaction of treatment, parity and DIM was also significant. During the differential management period, no differences in milk production were observed between treatments in primiparous cows, while T21 multiparous cows produced more milk than T0 multiparous cows. Following the shift to grazing, T21 primiparous cows exhibited lower milk production than T0 primiparous cows between 28 and 42 DIM, and then milk production converged between treatments (Fig. 1A). In T21 multiparous cows, milk production dropped following the management change, reaching levels comparable to those of T0 multiparous cows and remained stable until the end of the study (Fig. 1B). Multiparous cows produced more milk solids than primiparous cows (4.94 vs 3.65 kg/cow/day, respectively, Table 1). The triple interaction was significant (Table 1), as primiparous T21 cows showed lower total solids than primiparous T0 cows at 42 DIM (Fig. 1C), while in multiparous cows T21 yielded more milk solids than T0 at 7 and 14 DIM (Fig. 1D).

There was no significant effect of treatment on the days to resumption of ovarian cyclicity (32.7 ± 2.7 and 31.1 ± 3.1 days, for T0 and T21, respectively; P = 0.9038). Parity had a significant effect as primiparous showed a lower hazard of resumption than multiparous cows (HR = 0.42; P = 0.0380, Fig. 1E, F). Multiparous cows exhibited an earlier resumption of ovarian cyclicity (28.5 ± 2.2 days) compared to primiparous cows (36.4 ± 3.8 days).

Body condition score, metabolites and hormones

The BCS was affected by treatment, as T21 cows showed a greater BCS on average than those in the T0 group, and BCS decreased over DIM (Table 1). The T0 cows showed a greater ΔBCS at 21 DIM compared to T21 cows (-0.22 ± 0.04 vs -0.10 ± 0.04, P = 0.0280), and tended to have a greater ΔBCS at nadir than T21 cows (-0.53 ± 0.05 vs -0.40 ± 0.05, respectively, P = 0.0683). The ΔBCS at nadir was lower for primiparous than multiparous cows (-0.37 ± 0.06 vs -0.56 ± 0.05, P = 0.0368) and primiparous also reached the nadir earlier (31.5 ± 4.7 days) than multiparous cows (50.9 ± 3.9 days; P = 0.0110).

The concentrations of NEFA were significantly affected by the treatment, with T0 cows exhibiting greater concentrations than T21 cows (Table 1). Additionally, NEFA concentrations were significantly affected by the triple interaction of treatment, parity, and DIM. In T0 primiparous cows, NEFA concentrations were greater than those of T21 primiparous cows during the first 21 DIM (Fig. 2C). On the other hand, multiparous cows showed no differences in NEFA concentrations until 15 DIM, after which T21 multiparous cows exhibited a more rapid decline, resulting in lower concentrations than T0 multiparous cows from 18 to 21 DIM (Fig. 2D). In the first week after the management change (day 25), T21 primiparous cows had greater NEFA concentrations than T0 primiparous cows, with no differences observed thereafter. In multiparous cows, no differences in NEFA concentrations were detected between treatments following the management change. However, T21 multiparous cows experienced a rise in NEFA concentrations between days 25 and 30, surpassing prepartum levels, with no differences observed thereafter. Concentrations of BHB were significantly affected by the triple interaction of treatment, parity, and DIM (Table 1). No differences were observed in primiparous cows between treatments, while after the transition to grazing T21 multiparous cows exhibited greater BHB concentrations than T0 multiparous cows (Fig. 2F).

Cholesterol concentrations were affected by the triple interaction (Table 1). Although both treatments exhibited similar profile evolution, cholesterol levels were greater in T0 cows at 21 DIM for primiparous (Fig. 2G), and between 18 and 25 DIM for multiparous cows (Fig. 2H), compared to their parity counterparts in the T21 group.

Greater insulin concentrations were found in T21 cows compared to those in the T0 group, and in primiparous compared to multiparous cows (Table 1). The interaction between treatment and parity was significant ($P = 0.0007$). Average insulin concentrations were greater in T21 primiparous cows than in T0 primiparous cows (21.8 ± 1.3 vs 13.8 ± 1.23 ng/mL; $P < 0.0001$), and in T21 multiparous compared to T0 multiparous cows (12.3 ± 1.0 vs 10.1 ± 1.2 ng/mL; $P = 0.0482$). Insulin concentrations was also affected by the interaction among treatment, parity and DIM (Table 1). While T21 primiparous cows had greater insulin concentrations during the first 21 DIM compared to T0 primiparous cows, with similar values thereafter (Fig. 3A), T21 multiparous cows showed greater insulin concentrations at 3 and 9 DIM but reached comparable levels to T0 multiparous cows thereafter (Fig. 3B). This interaction also revealed differences between parities within treatment, as primiparous cows in T21 had consistently greater insulin concentrations than multiparous T21 cows across most days. In contrast, no significant differences between parities were observed over time in the T0 group.

On average, greater IGF-1 concentrations were found in T21 cows compared to T0, and in primiparous compared to multiparous cows (Table 1). The concentration of IGF-1 was affected by the interaction among treatment, parity and DIM (Table 1). Both primiparous (Fig. 3C) and multiparous (Fig. 3D) T21 cows had greater IGF-1 concentrations from 3 to 21 DIM compared to their counterparts in T0, with no differences thereafter. In both treatments, primiparous cows showed consistently greater IGF-1 concentrations than multiparous over time.

Serum metabolomics

To identify potential clustering based on treatment, time point, and parity, a PCA was performed as an unsupervised classification method using the data obtained from the ^{1}H NMR spectra of all serum samples, including quality controls. The score plot suggested a separation between treatments in primiparous cows at 21 DIM (Fig. 4A). In contrast, the distinction between treatments was not clear in multiparous cows at 21 DIM (Fig. 4B), as well as for primiparous (Fig. 4C) and multiparous cows (Fig. 4D) at 60 DIM.

These results were further confirmed using supervised classification (OPLS-DA), which showed a clear divergence between treatments only in primiparous cows at 21 DIM (Fig. 5A). The accuracy of discrimination was tested, yielding an AUC of 0.96 in the ROC analysis, and was also statistically significant in the permutation test. The corresponding loading plot indicated that glucose and valine were more abundant in T21 primiparous cows, whereas urea, acetoacetate, BHB, lactate, glycine, dimethylglycine, choline, betaine, creatine, creatinine, alanine, acetate, and lipids were present at greater concentrations in T0 primiparous cows (Fig. 5B). Although the OPLS-DA model for multiparous cows at 21 DIM showed a similar trend (Fig. 5C, D), it had a lower AUC (0.77) and did not pass permutation test validation. Likewise, the OPLS-DA models for both parity groups at 60 DIM failed to pass the permutation tests, with AUC values of 0.71 and 0.61 for primiparous and multiparous cows, respectively. After conducting the univariate analysis based on the relative concentrations of individual metabolites obtained from spectral integration, metabolites affected by the treatment, or its interaction were included for description. The relative concentrations of glucose differed according to the interaction between treatment and parity (Table 2), as they were greater in T21 than in T0 primiparous cows but showed no differences for multiparous cows (Figure 6A, B). Also, primiparous T21 cows showed greater glucose concentrations than multiparous T21 cows, but no differences between parities were found in T0 cows. The interaction between parity and day was significant, as glucose concentrations were lower for multiparous than primiparous cows at 60 DIM, without differences between parities at 21 DIM. The relative concentrations of BHB differed according to the interaction between treatment and day, as T0 cows had greater concentrations than T21 cows at 21 DIM (P = 0.0215, Fig. 7C), while T21 cows at 60 DIM showed greater concentrations of this metabolite than T0 cows (P = 0.0378). Acetate differed according to the interaction between treatment and parity, as its relative concentration in the T21 group was greater in multiparous than in primiparous cows (P = 0.0029), without differences between parities in T0 cows. Valine and histidine concentrations were affected by the interaction between treatment and day, as T21 cows had greater concentrations of these amino acids than T0 cows at 21 DIM, but no differences were found at 60 DIM. Creatinine concentrations were greater in T0 than T21 cows and in primiparous compared to multiparous cows. A significant interaction between treatment and day was observed, with T0 cows showing greater creatinine concentrations than T21 cows at 21 DIM (P = 0.001), without differences between treatments at 60 DIM. Creatinine concentrations increased from 21 to 60 DIM in T21 cows (P = 0.0009), whereas no changes over time were observed in T0 cows. A tendency in the interaction between treatment and parity was detected, as creatinine concentrations were greater in primiparous T0 than in primiparous T21 (P = 0.0049) and multiparous T0 cows (P = 0.0028). However, no differences were found between treatments in multiparous cows or between parities in T21 cows. Allantoin concentrations tended to be greater in T0 than T21 cows (Table 2). There was a tendency for the interaction between treatment and day as T0 cows had slightly greater concentrations of this diureide than T21 animals at 21 DIM (P = 0.0563). Allantoin concentrations tended to decrease from 21 to 60 DIM in T0 cows (Table 2). The interaction between parity and day was significant as multiparous showed greater allantoin concentrations than primiparous cows at 21 DIM (P = 0.0032), decreasing at 60 DIM compared to their 21 DIM levels (P = 0.0039). Dimethylglycine concentrations tended to be greater in T0 than in T21 cows. There was a significant interaction between treatment and parity, as primiparous T0 had greater concentrations of this metabolite than both primiparous T21 (P = 0.0028) and multiparous T0 (P = 0.0049). Formate concentrations were greater in T0 than in T21 cows. There was a tendency for the triple interaction, as at 21 DIM, primiparous T0 cows had greater formate concentrations than primiparous T21 (P < 0.0001, Fig. 7P) and also compared to multiparous T0 (P = 0.0043), decreasing at 60 DIM compared to their 21 DIM levels (P < 0.0001).

Discussion

To the best of our knowledge, this is the first study to investigate the parity-specific responses in dairy cows to a differential feeding management (indoor-fed TMR ad libitum for the first 21 days postpartum vs grazing with PMR supplementation after calving) in terms of endocrine-metabolic adaptation to lactation which links to productive and reproductive indicators. While TMR-confinement yielded a 12% greater milk production in multiparous cows, no differences in milk production were detected in primiparous cows during the first 21 DIM. However, the effect of the treatment on the endocrine-metabolic indicators was more marked in primiparous than in multiparous cows. The resumption of ovarian cyclicity was not affected by treatment, but multiparous cows had a shorter anestrus than primiparous cows. Thus, the present results suggest that strategic feeding management during the fresh cow period elicits different metabolic and productive responses depending on parity.

In the same experiment but under two calving seasons, we have reported that this dietary intervention did not enhance the productive performance of primiparous cows and suggested that an extended TMR period may be needed to reveal their full productive potential (Rivoir et al., 2025). Indeed, greater milk production in primiparous cows under TMR vs similar pasture-based systems was observed only after 35 DIM (Meikle et al., 2013). For multiparous cows, the differences between treatments were detected already at the first week postpartum, averaging 12% greater milk production in T21 compared to T0 multiparous cows during the first 21 days postpartum. This response aligns with reports of up to 15% greater milk production in Holstein multiparous under similar feeding regimes during early lactation (Fajardo et al., 2015; Mendina et al., 2024), and increases of 5–20% during mid-lactation (Bargo et al., 2002; Mendoza et al., 2016; Salado et al., 2018, 2020). Previously, two studies evaluated short-term TMR-confinement management during the first 21 (Al Ibrahim et al., 2013) or 30 days postpartum (Brady et al., 2021), followed by two grazing sessions and concentrate supplementation. Al Ibrahim et al. (2013) included only multiparous cows, whereas Brady et al. (2021) adjusted parity in the model as a covariate. In these studies, no differences were found in terms of milk production, which has been attributed to the small magnitude of differences in DMI, as well as the lower starch and CP content of the TMR. Nonetheless, Brady et al. (2021) suggested a carryover effect on milk production as it tended to be greater in TMR-fed cows after the change of management.

In the present study, the T21 management was insufficient in generating carryover effects on milk yield after the shift to grazing, which could be attributed to the short duration of the feeding treatment (see review: Jørgensen et al., 2016) and the greater milk production achieved during the differential period. Despite the drop in milk production when switching to pasture plus PMR, the multiparous T21 cows were able to adapt and maintain their milk production at the same levels as cows that were already adapted. Similarly, no differences were observed in a longer intervention (first 10 weeks postpartum), when multiparous Holstein cows fed TMR ad libitum were switched to grazing, compared to cows that grazed from calving (Fajardo et al., 2015). In the present study, as in Fajardo et al. (2015), high levels of PMR were offered, which combined with favorable pasture conditions, enabled cows to maintain a high DMI. This contrasts with other studies where the transition to grazing, along with reduced supplementation, was accompanied by marked declines in production (Schären et al., 2016; Hartwiger et al., 2018). In primiparous cows, the shift from TMR to grazing, resulted in a maintenance of the milk production, while T0 primiparous cows continue to increase milk production as part of the physiological lactation curve. This plateau in T21 cows led to an 11% difference in milk yield during the first three weeks following the management change. After that, milk production was no longer different between T21 and T0 primiparous cows, coinciding with the time for behavioral, metabolic, and ruminal adaptation to the shift from TMR to grazing previously reported (Schären et al., 2016, 2017).

Regarding metabolic indicators, the similar NEFA concentrations in multiparous T21 and T0 cows in the first two weeks postpartum suggest that the greater milk production observed in T21 was sustained by the extra dietary energy. On the other hand, the lower NEFA concentrations in primiparous T21 cows compared to T0 cows –and the similar milk production mentioned above– is consistent with the better energy status due to the TMR diet. These findings align with the reported lower capacity for nutrient partitioning towards milk production in primiparous cows (Wathes et al., 2006; Friggens et al., 2007; Morales Piñeyrúa et al., 2018; Ruprechter et al., 2018), in contrast to its prioritization in multiparous cows (Wathes et al., 2006, 2007). This aligns with BCS findings, as primiparous cows showed a lower loss of BCS and earlier nadir than multiparous cows, which likely prioritized the partitioning of body reserves to support milk production over a longer period (Wathes et al., 2006; Friggens et al., 2007; Morales Piñeyrúa et al., 2018; Ruprechter et al., 2018). Nevertheless, the mobilization phase was still shorter for T21 than T0 multiparous cows, as evidenced by the lower NEFA concentrations by 18–21 DIM in the former. Both parities in T21 experienced a metabolic readaptation when starting to graze, which was reflected by the increase of NEFA and BHB concentrations. This finding could be attributed to the lower DMI and energy density of the diet, as well as to the new energy expenditure in walking and grazing activities (Kolver and Muller, 1998; Bargo et al., 2002), and behavioral and social adaptation (Chilibroste et al., 2012). However, multiparous T21 cows maintained greater BHB concentrations until the end of the study compared to T0 multiparous cows, which seemed to be decreasing the activity of ketogenic pathways in this period.

Near the end of the differential management period (18 to 21 DIM), T0 cows exhibited greater cholesterol concentrations compared to T21 cows. Cholesterol profiles in this stage have been described as somehow controversial (Cavestany et al., 2005). Positive associations with dry matter intake have been reported (Drackley et al., 2014; Mendina et al., 2024), but unexpected lower concentrations of cholesterol in TMR-fed cows have been attributed to nutrient-specific characteristics of the diet (Meikle et al., 2013). The elevated cholesterol concentrations in T0 cows could be attributed to the greater ether extract content in the pasture and PMR compared to the TMR used in this study (Supplementary Table 1 and 2). This difference appeared earlier and persisted longer in multiparous T0 than in primiparous cows, likely due to the greater intake capacity of the former animals.

The greater glucogenic sources in TMR-based diets were evidenced by the greater insulin and IGF-1 concentrations during the differential feeding management period in both parities of T21 compared to T0 cows (Kolver and Muller, 1998; Lucy et al., 2013). Insulin and IGF-1 levels were greater over time in primiparous than in multiparous T21 cows, in line with the literature (Taylor et al., 2004; Wathes et al., 2007; Morales Piñeyrúa et al., 2018; Ruprechter et al., 2020; Cattaneo et al., 2023), and the uncoupling of the somatotrophic axis in favor of milk production in multiparous cows (Bauman and Currie, 1980; Wathes et al., 2007). However, the difference in insulin concentrations between parities was not expressed in T0 cows over time. Insulin has been proposed as a more immediate indicator of daily nutrient intake, whereas IGF-1 profiles better reflect changes in BCS (Adrien et al., 2012). In this context, despite being raised on pasture, primiparous cows seemed to struggle more with grazing behavior (Chilibroste et al., 2012), potentially explaining their comparable insulin concentrations along time to those of multiparous cows in the T0 group. These findings could suggest differences between parities adapting to reach their potential DMI under grazing conditions, in line with Chilibroste et al. (2012). Unfortunately, in the present study we do not have precise measurements of individual pasture and PMR intake and can thus only speculate based on PMR group feeding records and pasture intake through energy balance or pasture disappearance estimations.

Even with several endocrine-metabolic differences between treatments in primiparous cows, no effect on cyclicity was detected in this parity group. It has been suggested that primiparous cows are more sensitive to endocrine-metabolic signals which can delay the resumption of ovarian cyclicity (Santos et al., 2009). Factors limiting earlier reproductive stimulation may include the brief treatment duration before peak lactation and maximum feed intake capacity (Ingvartsen and Andersen, 2000), and the metabolic re-adaptation after switching to grazing. Although it potentially alleviates the NEB in the first three weeks postpartum, the feeding management was insufficient to impact the resumption of ovarian cyclicity in both primiparous and multiparous cows. This is consistent with reports indicating that early lactation NEB and nutritional strategies have limited effects on reproductive outcomes, as nutrients are primarily directed toward milk production (De Vries and Veerkamp, 2000; Butler, 2014; Berry et al., 2016). Nevertheless, and in agreement with previous findings (Meikle et al., 2004; Santos et al., 2009; Adrien et al., 2012; Ruprechter et al., 2020), multiparous cows exhibited a shorter postpartum anestrous period compared to primiparous cows. This difference is likely attributable to the additional growth requirements of primiparous cows (Coffey et al., 2006).

Serum metabolomic analyses revealed more significant differences between treatments for primiparous cows at 21 DIM, than for multiparous cows. Multivariate analyses did not detect significant differences between treatments at 60 DIM in either primiparous or multiparous cows. Overall, the OPLS-DA loading plot for primiparous cows at 21 DIM is consistent with insulin data, indicating greater circulating glucose in T21 cows, while markers of lipid and protein mobilization were more abundant in T0 cows.

Consistently, univariate analysis revealed that glucose concentrations differed between treatments in primiparous cows, but not in multiparous cows. This finding aligns with milk production and endocrine-metabolic profile results, as primiparous T21 cows did not respond to the feeding management with increased milk production but rather exhibited improved energy balance indicators. Conversely, multiparous T21 cows likely directed the extra circulating glucose towards the mammary gland to support greater milk yield, which could explain the lack of glucose differences between multiparous T0 and T21 cows despite the dietary contrast. Similar to insulin profiles, relative glucose concentrations did not differ between primiparous and multiparous cows in the T0 group but did in the T21 group. The relative glucose concentrations observed at 21 DIM declined at 60 DIM, suggesting a greater glucose uptake to meet the greater milk production (Lucy et al., 2014).

Metabolomics data also detected greater BHB levels in T0 cows at 21 DIM, despite no differences were detected by spectrophotometry on that day (Fig. 2E and F). These data is consistent with greater NEFA concentrations in T0 cows and reflect the greater NEB. Interestingly, BHB concentrations at 60 DIM were greater in T21 cows, which is consistent with BHB profiles at this time and, as mentioned before, could be the result of increased energy expenditure and nutrient intake levels upon transitioning to grazing (Kolver and Muller, 1998; Meikle et al., 2013). The lower relative concentration of acetate observed in primiparous T21 compared to T21 multiparous cows could reflect the greater DMI capacity in the later, with a greater influx from the rumen (Drackley et al., 2014), while this difference was not expressed in grazing cows with a greater proportion of acetate precursors (Ishler et al., 1996).

The greater concentrations of the glucogenic amino acids valine and histidine observed in T21 cows at 21 DIM may indicate a better protein balance status (Jorge-Smeding et al., 2021), which aligns with their improved energy status at this stage compared to T0 cows. Moreover, there was a decrease in both amino acids at 60 DIM in T21 cows, suggesting the limitation of the grazing plus PMR diet to maintain high milk production through its contribution to lactose and milk protein synthesis.

The level of creatinine, an indicator of body muscle mass and muscle protein breakdown (Megahed et al., 2019; Sadri et al., 2023), was greater in T0 than in T21 cows at 21 DIM, a difference mainly originated from the greater concentrations of this metabolite in primiparous T0 cows. Data suggest that primiparous T0 cows mobilized protein reserves to sustain similar milk production levels than T21 cows (Megahed et al., 2019). The greater creatinine levels in grazing could also be due to the higher physical activity on walking and grazing. Our results also showed greater concentrations of creatinine for primiparous T0 compared to multiparous T0 cows, but this difference was not detected for T21 cows. Overall, it seems reasonable to suggest that, under grazing conditions, primiparous cows struggle to meet their metabolic requirements during early postpartum (as further indicated by their lower glucose concentrations), leading to increased muscle mobilization. Allantoin was greater in T0 than in T21 cows at 21 DIM, while no differences were observed at 60 DIM. This metabolite has been linked to oxidative stress in cattle (Liao et al., 2018) and humans (Kand’ár and Žáková, 2008), which may be particularly relevant during the immediate postpartum period (Sordillo and Mavangira, 2014). Moreover, allantoin is also associated with triglyceride metabolism and has been reported to be greater in cows with subclinical ketosis (Wang et al., 2016), which, as previously described, may better align with the metabolic profile of T0 cows at this time.

Dimethylglycine and formate relative concentrations were greater in T0 vs T21 primiparous cows. Dimethylglycine is generated as a by-product in the conversion from betaine to methionine. This process releases a one-carbon unit, which can be oxidized to formate, contributing to the one-carbon pool for nucleotide synthesis and methylation reactions (McFadden et al., 2020). Dairy cows are unable to synthesize methionine in sufficient quantities to meet the demands for fetal growth and milk protein synthesis (Arshad and Santos, 2024). With the conversion from betaine to methionine, cows rely heavily on folate metabolism to support methylneogenesis during the peripartum period and early lactation (Xue and Snoswell, 1986), which may explain the greater abundance of both dimethylglycine and formate observed in primiparous T0 cows, particularly at 21 DIM, coinciding with their more pronounced NEB. Although knowledge of one-carbon metabolism in dairy cows remains limited, the marked differences observed in these metabolites reinforce their relevance not only in milk production, but also in hepatic health and immune response, making this a growing area of interest as previously reported (McFadden et al., 2020).

In conclusion, strategic feeding management during the fresh cow period elicits distinct endocrine-metabolic and productive responses, reflecting parity-specific homeorhetic priorities. Feeding TMR ad libitum during the first 21 days postpartum allowed primiparous cows to preserve body reserves without increasing milk yield, whereas primiparous cows managed under grazing and supplemented with PMR after calving appeared to struggle to meet their metabolic requirements, relying on greater lipid and muscle mobilization. In contrast, multiparous cows fed TMR exhibited greater milk production and similar lipid mobilization levels in the first two weeks postpartum compared to their counterparts in a pasture-based system after calving. The transition from TMR to grazing management triggered a metabolic re-adaptation in both parities, and no carryover effect on milk production was observed. Despite the productive and metabolic benefits, the differential fresh cow feeding strategy with TMR during first 21 days postpartum did not influence the time to the resumption of ovarian cyclicity.

Notes

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Author ORCIDs

G.R. Mendina: https://orcid.org/0000-0003-1311-4421

M.L. Adrien: https://orcid.org/0000-0001-5914-1981

C. Rivoir: https://orcid.org/0000-0001-8269-0815

A. López: https://orcid.org/0000-0003-0203-0732

G. Moyna: https://orcid.org/0000-0002-0727-1079

P. Chilibroste: https://orcid.org/0000-0001-9579-9967

A. Meikle: http://orcid.org/0000-0002-0847-8629

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Appendix

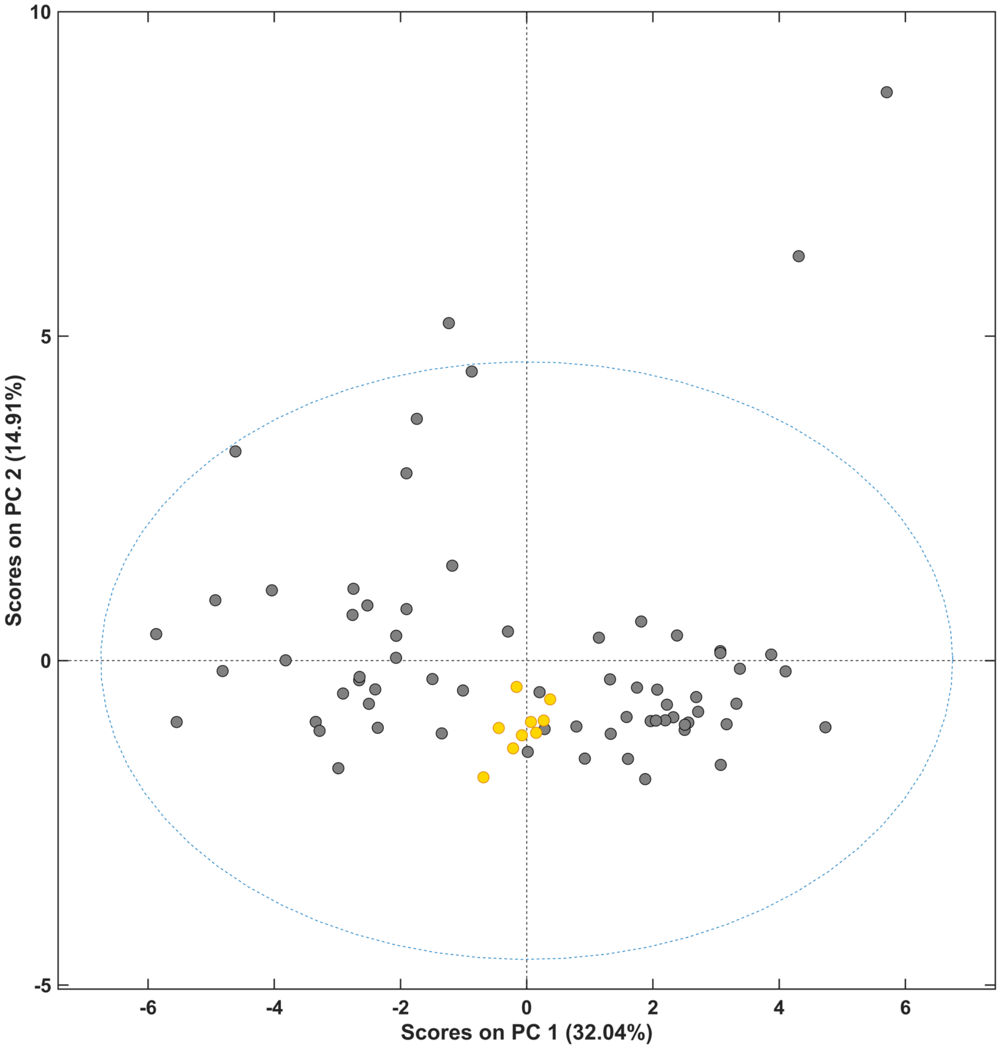


Figure A1. PCA score plot obtained from ^{1}H-NMR spectral data of serum samples collected at 21 and 60 DIM from primiparous and multiparous cows in T0 and T21 groups, and quality control (QC) samples (yellow circles).

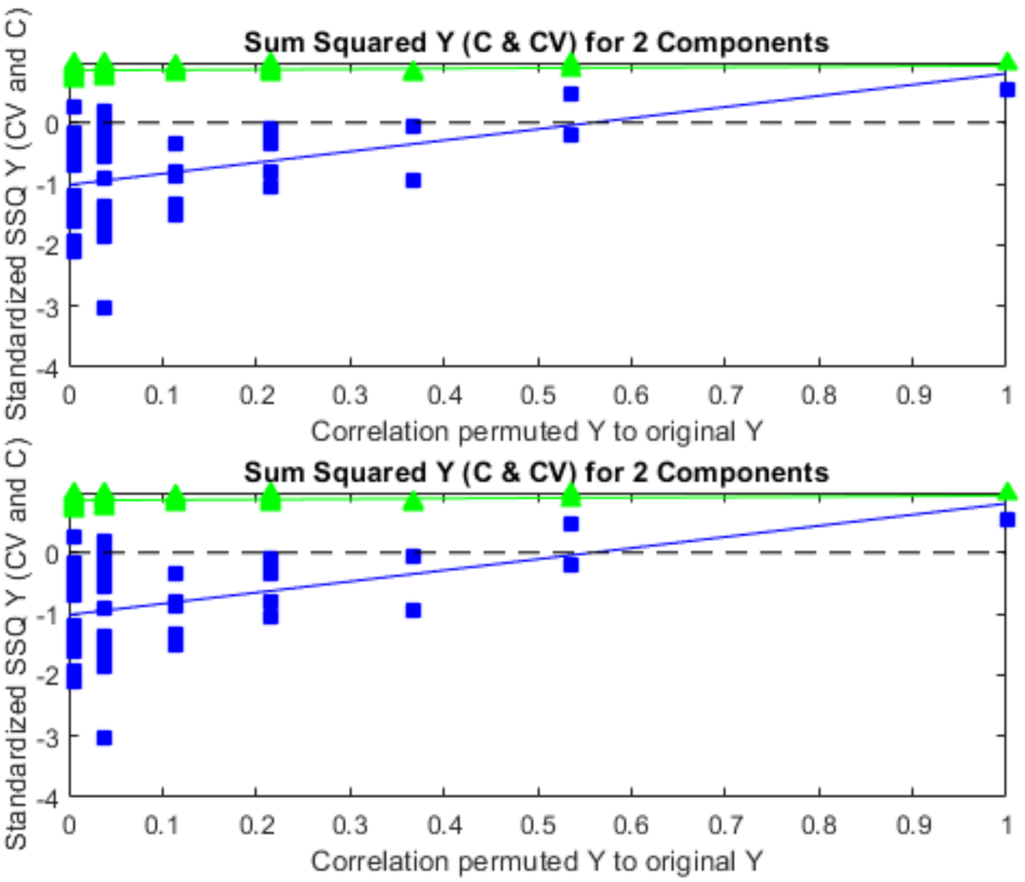


Figure A2. Permutation test plots for the OPLS-DA model comparing primiparous T0 and T21 at 21 DIM (R2Y = 0.92 and Q2Y = 0.48).

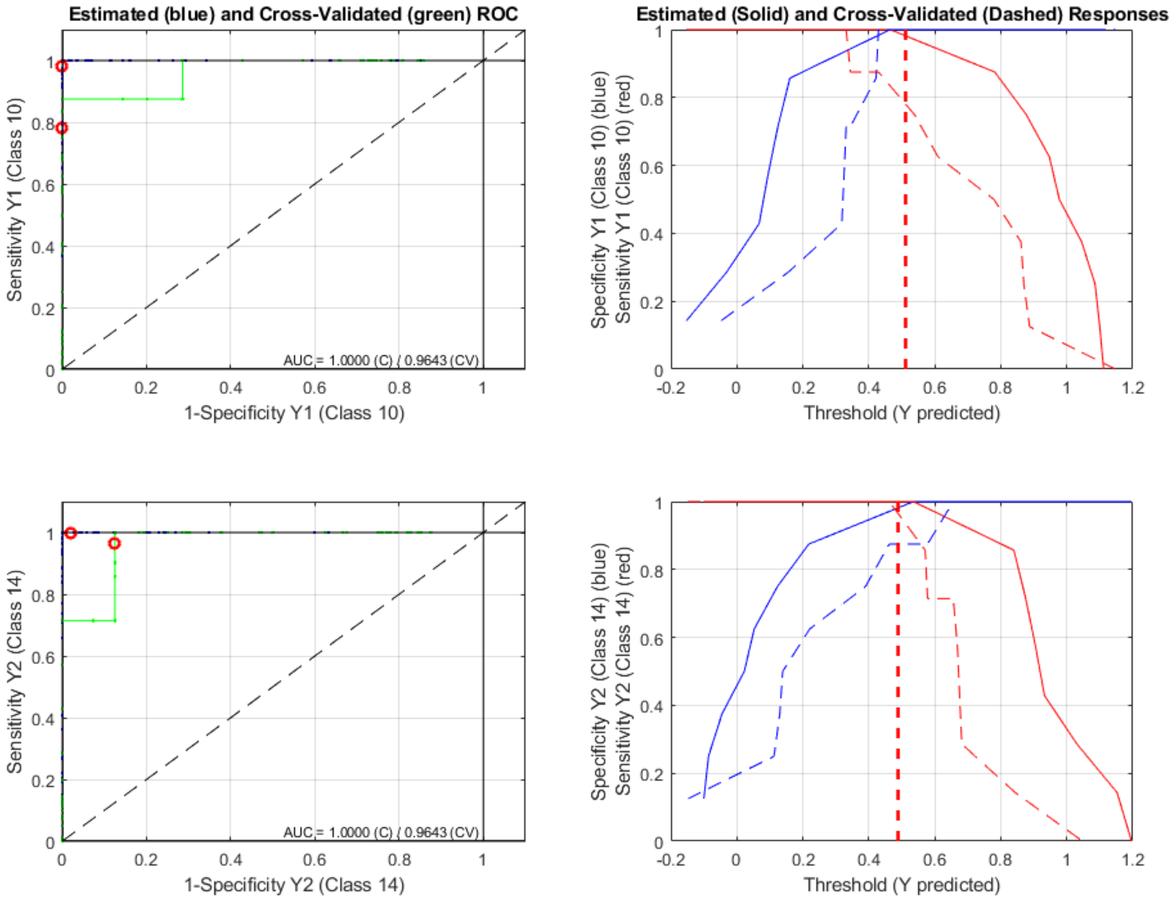


Figure A3. ROC analysis curves derived from the OPLS-DA model comparing primiparous T0 and T21 at 21 DIM (AUC = 0.96).

Table A1. Ingredient composition (% of DM) of TMR and PMR.

Treatments	T0	T21	T0 & T21
Period	0-21 DIM		22-60 DIM
Ingredients, % of DM			
Corn silage	15	44.3	16.4
Sorghum silage	27.6	-	24.3
Moha hay	1.7	4.6	-
Corn grain	19.1	17.4	21
Soybean meal	10.5	11.7	7.2
Canola meal	-	5.8	-
Sunflower expeller	-	5.8	-
Soybean hulls	-	7.7	-
Wheat bran	22.7	-	24.1
Corn DDGS	-	-	3.1
Minerals and vitamins	3.7	2.5	3.9
Forage:concentrate ratio	44:56	49:51	41:59

Abbreviations: DM: Dry matter, DDGS: Dried distillers grains with soluble.

Table A2. Chemical composition and offer of TMR (T21) and PMR (T0 and both treatments after 22 DIM)[1]

Treatments	T0	T21	T0 & T21
Period	0-21 DIM		22-60 DIM
% of DM[2]			
DM	40.1 ± 2.1	50.2 ± 2.4	45 ± 4.1
CP	14.3 ± 0.05	17.1 ± 0.3	14.3 ± 0.4
NDF	39.2 ± 1.6	36.5 ± 1.8	40.0 ± 1.7
ADF	17.6 ± 1.0	18.9 ± 1.6	17.4 ± 0.5
Ash	9.1 ± 0.1	7.5 ± 0.4	9.9 ± 0.9
Ether extract	2.4 ± 0.3	1.9 ± 0.1	2.5 ± 0.3
NEL (Mcal/kg DM)	1.64 ± 0.02	1.62 ± 0.05	1.65 ± 0.01
Offer (kg DM/d)*	13.3 ± 0.5	29.9 ± 3.5	13.1 ± 0.8

[1] Values expressed as the average ± the standard deviation.

[2] DM: Dry matter, PC: Crude protein, NDF: Neutral detergent fiber, FDA: Acid detergent fiber, NEL: Estimated net energy of lactation using NEL (Mcal/kg) = 2.149 − (0.0223 × ADF), (NRC, 2001).

* The amount of offered PMR corresponded to the intake, as indicated by the empty feeders in the morning.

Table A3. Characteristics and chemical composition of the pasture according to period[1]

Treatments	**T0**	**T0 & T21**
Period	**0-21 DIM**	**22-60 DIM**
Herbage allowance, kg DM/cow/d	27.6 ± 0.6	27.1 ± 4.5
Pre-grazing herbage mass, kg DM/ha	2090 ± 535	2128 ± 372
Post-grazing herbage mass, kg DM/ha	1533 ± 714	1481 ± 363
Pre-grazing sward height, cm	24.2 ± 7	21.1 ± 4.1
Post- grazing sward height, cm	13.5 ± 1.3	14.5 ± 1.3
% of DM[2]		
DM	23.7 ± 3.3	22.4 ± 4.1
CP	20.6 ± 4.3	18.7 ± 4.1
NDF	34.1 ± 7.1	36.1 ± 2.6
ADF	16.4 ± 3.0	17.6 ± 2.0
Ash	11.3 ± 1.4	11.3 ± 1.4
Ether extract	3.2 ± 0.7	3.2 ± 0.6
NEL (Mcal/kg DM)	1.66 ± 0.03	1.64 ± 0.03

[1] Values expressed as the average ± the standard deviation.

[2] DM: Dry matter, CP: Crude protein, NDF: Neutral detergent fiber, FDA: Acid detergent fiber, NEL: Estimated net energy of lactation using (2.301 − (0.0289 × %ADF)) × 4.1868 × 0.239 (Acosta, 2004).

942 Tables and Figures

Table 1. Milk production, total milk solids, metabolites and hormones for T0 and T21 treatments

	Treatments			P-value			
	T0	T21	SEM	Treat	DIM	Parity	Treat*Parity*DIM
Milk production, L/cow/day	34.6	34.7	1.2	0.9133	<0.0001	<0.0001	<0.0001
Total milk solids, kg/cow/day	4.2	4.4	0.2	0.5357	0.1144	<0.0001	0.0047
BCS[1], 1-5 scale	3.0	3.1	0.03	0.0370	<0.0001	0.6869	0.9027
NEFA[2], mmol/L	0.45	0.37	0.02	0.0209	<0.0001	0.3947	0.0376
BHB[3], mmol/L	0.58	0.60	0.02	0.3780	0.0003	0.2937	0.0201
Cholesterol, mmol/L	3.62	3.39	0.12	0.1048	<0.0001	0.9637	0.0497
Insulin, ng/mL	12.0	17.0	0.8	<0.0001	<0.0001	0.0005	<0.0001
IGF-1[4], ng/mL	68.7	80.7	3.2	0.0011	<0.0001	<0.0001	<0.0001

[1] Body condition score.

[2] Non-esterified fatty acids.

[3] Beta-hydroxybutyrate.

[4] Insulin-like growth factor – 1.

Table 2. Univariate analysis of metabolites relative concentration determined by NMR spectroscopy. Fixed effects are treatment, parity, day (21 vs 60 DIM), and their interactions

	P-value						
	Treat	Parity	Day	Treat x Parity	Treat x Day	Parity x Day	Treat x Parity x Day
α-glucose	0.0308	0.0118	<.0001	0.0076	0.9614	0.0307	0.5444
β-glucose	0.0128	0.0233	<.0001	0.0071	0.2553	0.0107	0.8755
BHB	0.8470	0.1330	0.8617	0.4175	0.0029	0.2676	0.6444
Acetate	0.9435	0.0419	0.4155	0.0256	0.3007	0.8465	0.1749
Valine	0.2840	0.7499	0.0020	0.8279	0.0393	0.0039	0.5739
Histidine	0.1648	0.8922	0.0008	0.7774	0.0007	0.0705	0.7252
Creatinine	0.0146	0.0068	0.0097	0.0731	0.0287	0.0889	0.7586
Allantoin	0.0559	0.0562	0.0931	0.9808	0.0915	0.0221	0.7703
Dimethylglycine	0.0640	0.1479	0.7705	0.0068	0.3526	0.0242	0.1790
Formate	0.0005	0.9768	0.0001	0.0813	0.0061	0.0078	0.0959

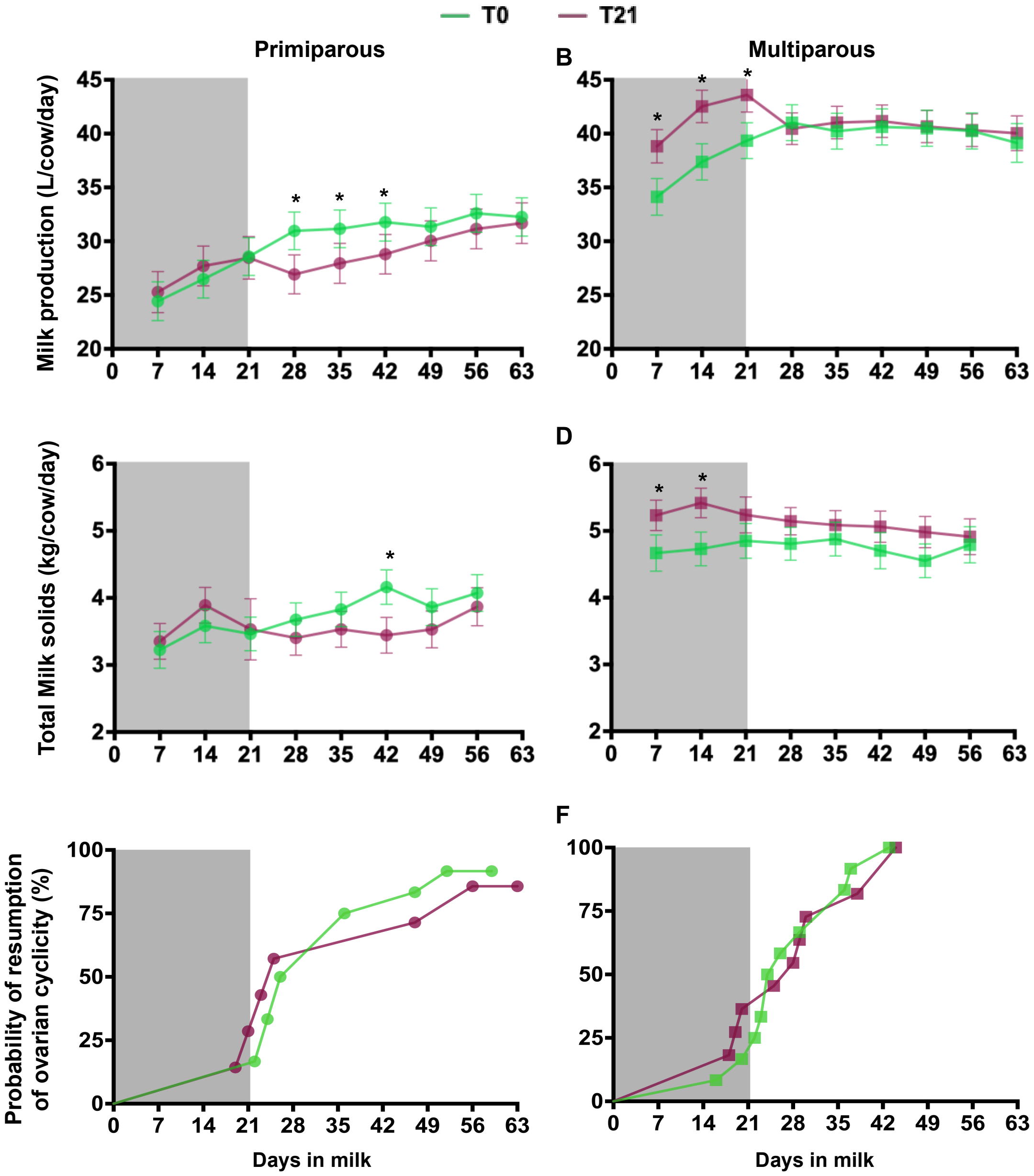


Figure 1. Milk production (L/cow/day; A, B), total milk solids (kg/cow/day; C, D), and survival curves of the probability of resumption of ovarian cyclicity (E, F) in the first 63 days in milk for primiparous (left) and multiparous (right) of T0 (green) and T21 (purple) cows. Grey area represents the period of the differential feeding management in T21.

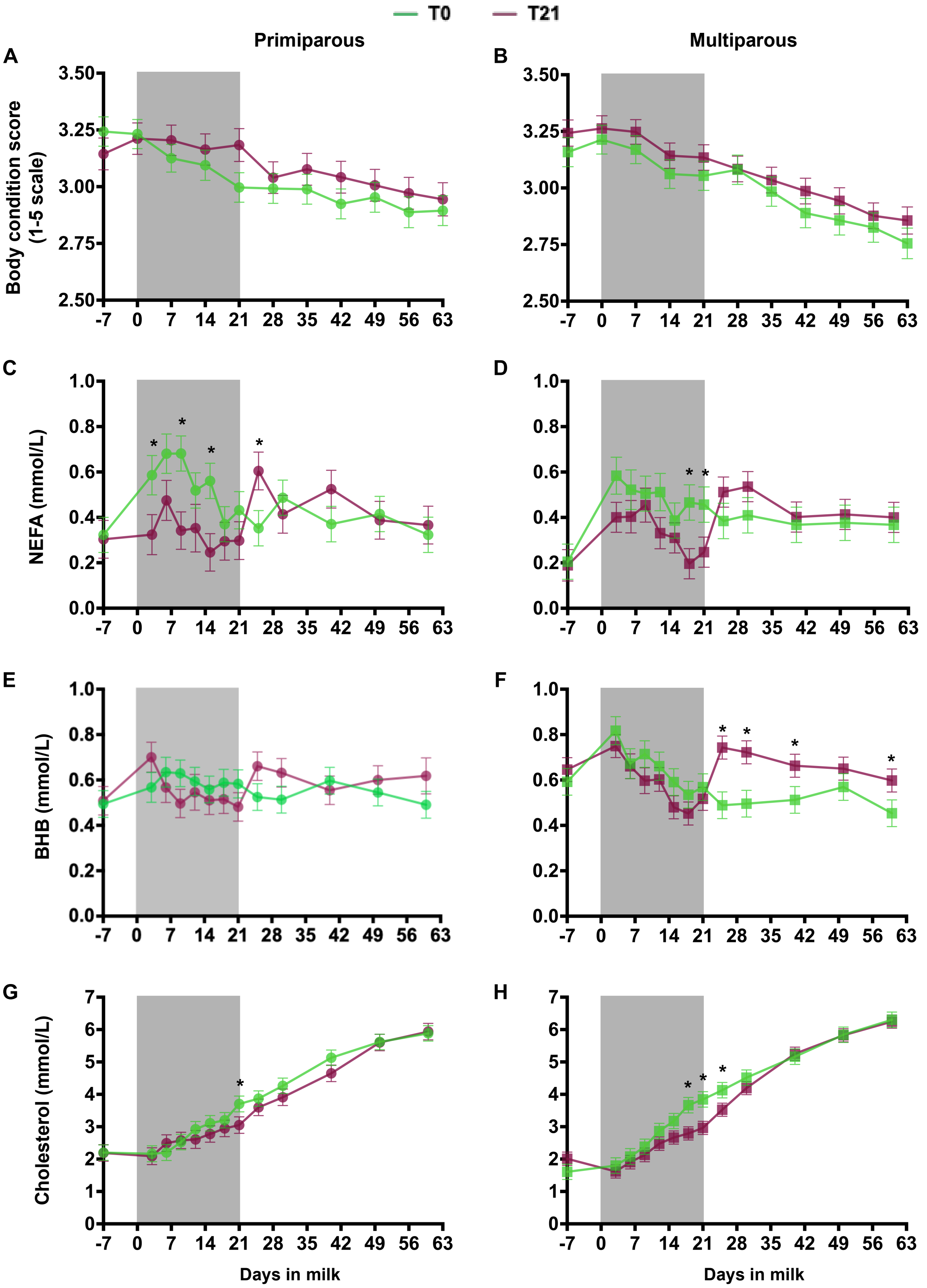


Figure 2. Body condition score (1-5 scale; A, B), non-esterified fatty acid (NEFA; C, D), beta-hydroxybutyrate (BHB; E, F), and cholesterol (G, H) concentrations in the first

60 days in milk for primiparous (left) and multiparous (right) of T0 (green) and T21 (purple) cows. Grey area represents the period of the differential feeding management in T21. Asterisks indicate significant differences between treatments.

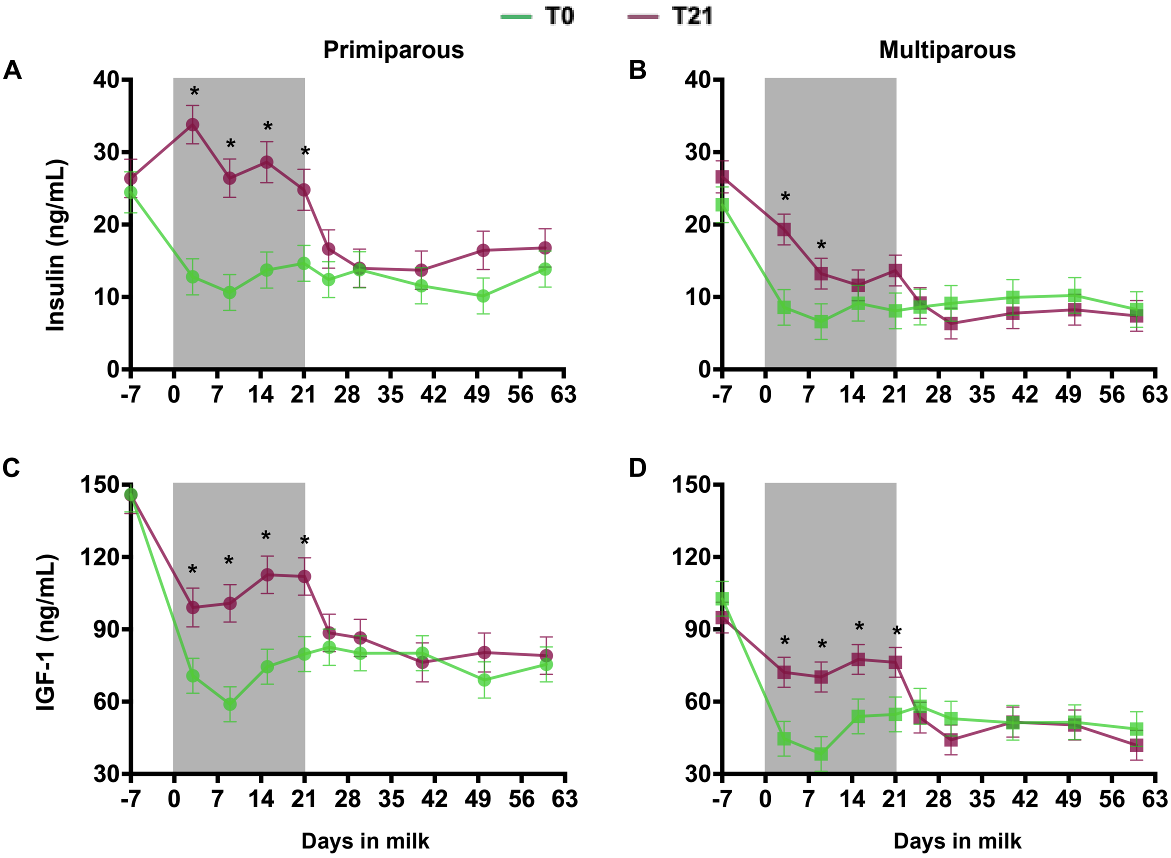
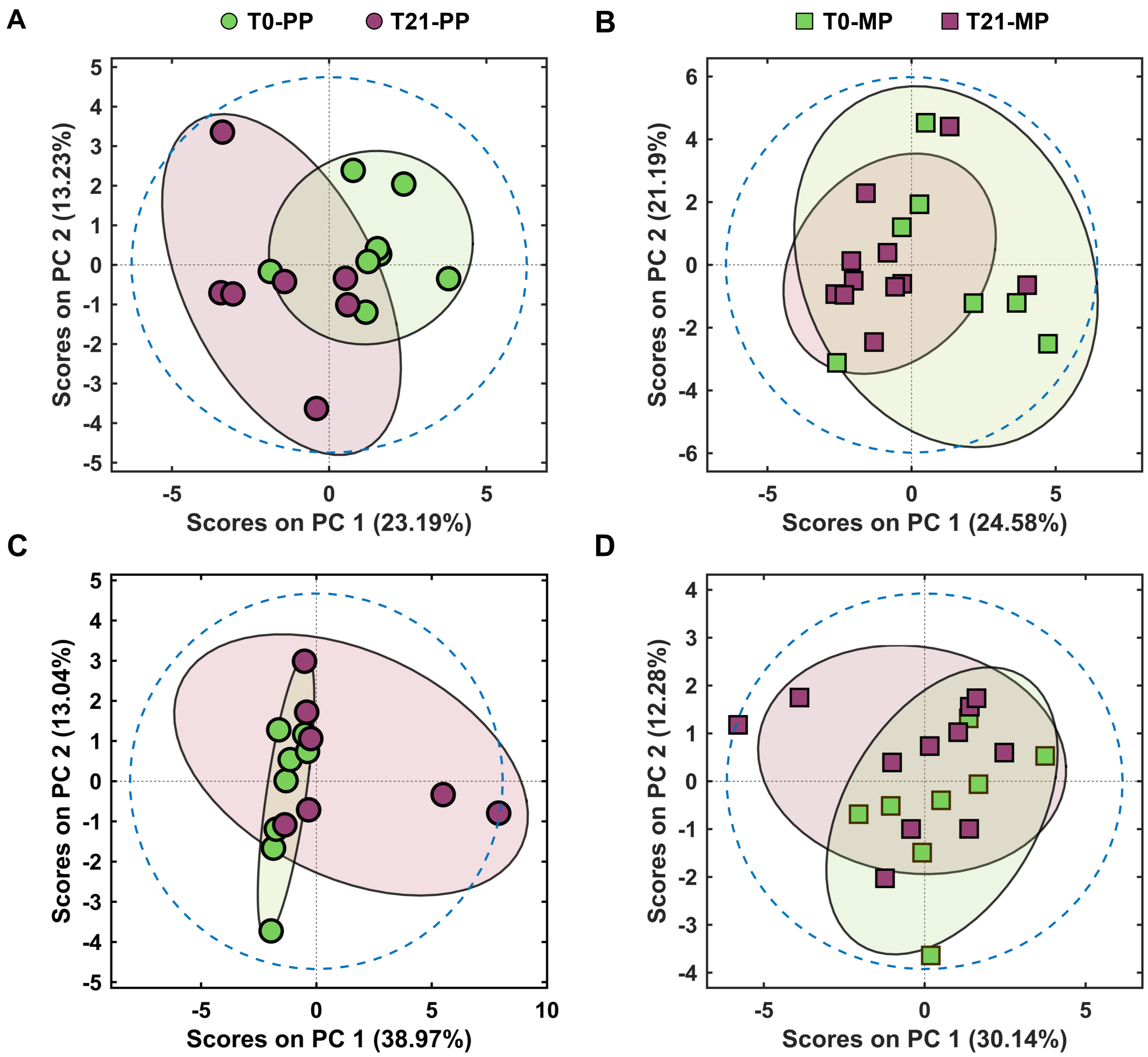


Figure 3. Insulin (A, B), and insulin-like growth factor (IGF-1; C, D) concentrations in the first 60 days in milk for primiparous (left) and multiparous (right) of T0 (green) and T21 (purple) cows. Grey area represents the period of the differential feeding management in T21.



975

976 Figure 4. PCA score plot obtained from ^{1}H-NMR spectral data of serum samples from

977 primiparous T21 (purple circles) and T0 (green circles) at 21 DIM (A) and 60 DIM (C)

978 and multiparous T21 (purple squares) and T0 (green squares) at 21 DIM (B) and 60

979 DIM (D).

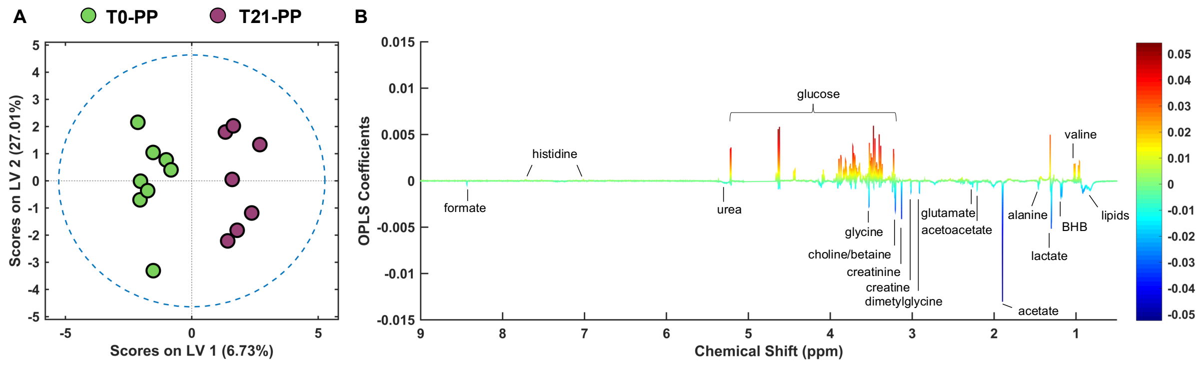


Figure 5. Score (A) and loading factor (B) plots obtained from the OPLS-DA between primiparous T21 and T0 cows at 21 DIM. The metabolites that differentiate the treatments are annotated in the loading factor plot. The R^2Y and Q^2Y coefficients were 0.92 and 0.48, respectively, and the ROC curve had an AUC value of 0.96 (see Figures A2–A3).

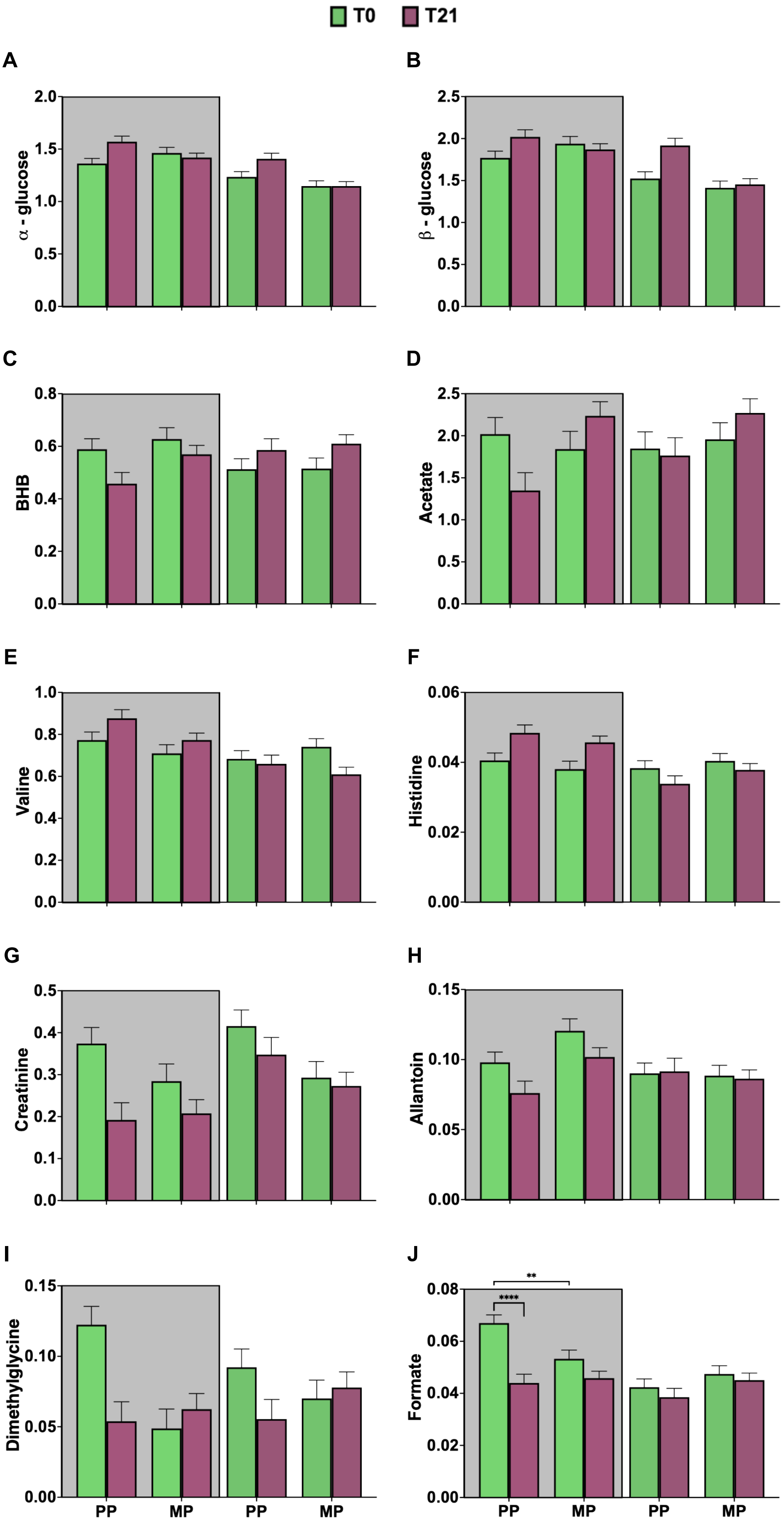

T0
T21
A
α - glucose
0.0
0.5
1.0
1.5
2.0
B
β - glucose
0.0
0.5
1.0
1.5
2.0
2.5
C
BHB
0.0
0.2
0.4
0.6
0.8
D
Acetate
0.0
0.5
1.0
1.5
2.0
2.5
E
Valine
0.0
0.2
0.4
0.6
0.8
1.0
F
Histidine
0.00
0.02
0.04
0.06
G
Creatinine
0.0
0.1
0.2
0.3
0.4
0.5
H
Allantoin
0.00
0.05
0.10
0.15
I
Dimethylglycine
0.00
0.05
0.10
0.15
J
Formate
0.00
0.02
0.04
0.06
0.08
**
****
PP
MP
PP
MP
PP
MP
PP
MP

Figure 6. Relative concentrations of serum metabolites in primiparous (PP) and multiparous (MP) of T0 and T21 groups at 21 (grey area) and 60 DIM. Data are presented as mean ± SEM. Asterisks indicate significant differences (**** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.01$, and * = $P \leq 0.05$).

Artículo III

Is there any residual effect of differential feeding strategies during fresh cow period on uterine transcriptomics and metabolomics at the end of the voluntary waiting period in the dairy cow? **Mendina GR**, Jara E, de Brun V, Adrien ML, Rivoir C, Chilibroste P, López-Radcenco A, Moyna G, Meikle A. Manuscrito.

Is there any residual effect of differential feeding strategies during the fresh period on endometrium transcriptomics and metabolomics at the end of the voluntary waiting period in the dairy cow?

Mendina et al.

Corresponding author: Graciana R. Mendina. E-mail: g.rmendina@gmail.com

Abstract

This study aimed to determine whether confinement with total mixed ration (TMR) during the first 21 days in milk (DIM), followed by grazing with partial mixed ration (PMR) supplementation (T21, n= 20), affects endometrial transcriptomic and metabolomic profiles at the end of the voluntary waiting period in primiparous (PP) and multiparous (MP) dairy cows, compared to a control group managed on pasture with PMR supplementation from calving (T0, n= 20). Cows were synchronized, and on day 7 of the estrous cycle (62 DIM), endometrial biopsies were collected for transcriptomic and metabolomic analyses. Only cows that showed estrus after synchronization, had a normal luteal phase, and met the quality criteria for the analyses were included (T0PP = 4, T21PP = 3, T0MP = 5, T21MP = 4). Progesterone levels determined daily from estrous were unaffected by treatment, though PP cows had higher concentrations than MP cows. RNA-Seq revealed modest transcriptomic differences, with 15 and 19 differentially expressed genes (DEGs) in PP and MP cows, respectively. In MP cows, T0 endometrium presented upregulation of genes involved in immune modulation, cellular renewal, and endocrine signaling (*IL17RB, FOS, FOSB, CYP19A1, PGFS*), along with higher relative lactate concentrations when compared to T21 MP cows. Conversely, T21 MP cows showed higher expression of *APOA5* and increased carnitine levels, markers of fatty acid uptake that may reflect an energetically stressed state. In PP cows, T0 PP cows showed higher expression of LPL and DEGs related to transcriptional and post-transcriptional regulation when compared to T21PP cows, possibly impacting the methylneogenesis pathway. The limited overall differentiation is associated with the similar management that the groups had for ~40 days before biopsy. Nonetheless, the DEGs identified showed biological relevance and reflect endocrine-metabolic and nutritional influences of the treatment imposed for the first 21 DIM and/or to the adaptation to grazing in T21 cows, which was parity dependent. Further research is warranted to clarify whether early lactation nutritional strategies have lasting effects on the uterine environment at breeding.

Keywords: Transition period, Endometrium, Parity.

Introduction

The uterine milieu at the time of breeding is a key determinant of reproductive success (Lonergan et al., 2019; Simintiras et al., 2019). The critical processes of establishing uterine receptivity depend on maternal systemic effects via the endometrium (Tinning et al., 2023). This condition is particularly sensitive in dairy cows, which undergo negative energy balance (NEB) during the transition period, while sustaining high milk production levels (Grummer et al., 2004). It has been proposed that the endometrium can sense the metabolic status of the cow (Meikle et al., 2018), as reflected in alterations in its transcriptome (Astessiano et al., 2017; Bauersachs et al., 2017). These modifications may, in turn, influence the composition of molecules secreted or transported into the uterine lumen, shaping the environment that supports early embryonic development (Fair, 2014; Bauersachs et al., 2017).

During the preimplantation stages, embryonic development relies on histotroph -the array of molecules secreted by the endometrium or transported into the uterine lumen, which serve as substrates and cofactors for multiple developmental processes (Roberts and Bazer, 1988; Binelli et al., 2022). Day 7 of the estrous cycle marks a key period of uterine remodeling and the onset of histotroph secretion (Moran et al., 2017), which becomes critical for sustaining the embryo after hatching and before placental attachment. It is estimated that 10–50% of pregnancy losses occur during the first week post-fertilization, with postpartum metabolic stress among the major contributing factors (Wiltbank et al., 2016).

Lactating multiparous cows with more severe NEB have an altered uterine transcriptome related to impaired remodeling and immune functions at two weeks postpartum (Wathes et al., 2009) or at the time of breeding (Valour et al., 2013). Consistently, Astessiano et al. (2017) reported a more favorable endometrial gene expression in primiparous cows fed TMR ad libitum compared to low herbage allowance plus supplementation cows, which showed the worst energy status. Several feeding strategies have been investigated to alleviate NEB, but studies focusing on short-term focalized strategies in the critical postpartum transition period are limited (Al Ibrahim et al., 2013; Brady et al., 2021; Rivoir et al., 2025). The response to these feeding strategies may differ depending on the parity of cows (Mendina et al., submitted), as nutrient partitioning and metabolic adaptation differ between primiparous and multiparous animals (Wathes et al., 2006). Nonetheless, the potential carryover effects of these short-term interventions on the uterine environment at the onset of the breeding period remain largely unknown.

This study hypothesizes that confinement with TMR ad libitum during the first 21 days in milk (DIM) enhances the uterine milieu at the time of the onset of the breeding period, compared to cows grazing with PMR supplementation from calving. Moreover, we propose that the response to this early postpartum management differs according to parity. Therefore, the aim of this study was to determine whether TMR-confinement during the first 21 DIM, followed by grazing with PMR supplementation, impacts endometrial transcriptomic and metabolomic profiles at the end of the voluntary waiting period in primiparous and multiparous dairy cows, relative to a control group managed on pasture with PMR supplementation from calving.

Material and methods

This study was performed at the Experimental Station Dr. Mario A. Cassinoni, Facultad de Agronomía, Universidad de la República, Paysandú, Uruguay (32° 23'07.6 "S 58° 03'17.9" W). The protocol was approved by the Ethics Committee of Universidad de la República, (Comité de Ética en el Uso de Animales de Experimentación, CEUA-CHEA ID 1344 - Exp. 020300-501632-21).

Animals and treatments

The experimental design of the present study has been described previously (Mendina et al. submitted). Briefly, the study was performed with 16 primiparous and 24 multiparous Holstein dairy cows that calved in winter. Before calving, cows were blocked according to the number of lactations, expected calving date, body weight (BW), and BCS, being then randomly distributed into two treatments: T0= grazing plus supplementation with PMR since calving, and T21= confinement with TMR *ad libitum* during the first 21 DIM and the same nutritional management of T0 from day 22 onwards. All the animals underwent a clinical examination by a veterinarian between 5 and 10 DIM, to check their health status and ensure their continuity in the experiment.

Because of calving complications or serious illnesses (caesarean section, downer cow syndrome, metritis), some animals were removed from the experiment. The final number of animals enrolled in the experiment included 15 primiparous (8 and 7 in the T0 and T21 groups, respectively) and 19 multiparous (8 and 11 in the T0 and T21 groups, respectively). The deviation of calving date was 25 days between first and last calving. The mean number of lactations of multiparous cows was 2.7±1.1, BW at calving was 566±48 kg for primiparous and 665±89 kg for multiparous, and BCS at calving was 3.2±0.17 and 3.3±0.24 for primiparous and multiparous cows, respectively. All animals had the same prepartum management in separate paddocks according to parity and were fed a TMR with anionic salts. During the postpartum period, primiparous and multiparous cows were managed together within each treatment, reflecting commercial practices. After calving, cows in T0 went out to graze between morning and afternoon milking (7:00 to 14:00h), and stayed in an outdoor soil-bedded yard, where the PMR supplementation was offered, during the rest of the day. Cows in T21 were housed in a compost-bedded pack barn during the first 21 DIM with *ad libitum* TMR, coming out only for milking. From 22 DIM onwards, cows of T21 joined T0 treatment and were managed together until approximately 60 DIM. Details of routine, housing and feeding management have been described in Mendina et al. (submitted).

Estrous cycle synchronization and sample collection

The estrous cycle was synchronized to facilitate biopsy collection with an objective of sampling on Day 7 of the estrous cycle (cows were not inseminated), which is an important stage for uterine remodeling and initiation of histotrophic secretion (Moran et al., 2017). This management was carried out at an average of 62 ± 5.4 days postpartum (mean±s.d.), as it is typically when cows are being bred after the voluntary waiting period. On days 40 and 51 postpartum, a synthetic prostaglandin analogue (D-cloprostenol, 0.075 mg/mL, Ciclar, Zoovet, Argentina) was administered to synchronize estrus. After the second injection, cows were fitted with estrus detection patches (Estrotect, Rockway Inc., USA). Cows were considered in estrus when more than 50% of the silver coating of the patch was removed and/or by visualization of standing to be mounted. Only healthy animals exhibiting estrus after synchronization, presenting a normal luteal phase, and meeting the quality standards for subsequent analyses were included in the study. Then, the final number of animals was primiparous: T0=4, T21=3; multiparous T0=5, T21=4.

Blood samples were collected from the coccygeal vein using an evacuated tube system (Vacuette 8 mL Serum Beads Clot Activator, Greiner Bio-One GmbH), starting 24 h (day 1) after the second injection of the prostaglandin analogue and on days 3, 5, 7, 8, 9, and 10. Blood samples were centrifuged at 1,680 × g for 10 min at room temperature, and serum was stored at −20°C until further analysis.

On day 10 after the second injection of the prostaglandin analogue, two samples of endometrial biopsies were collected from the uterine horn ipsilateral to the corpus luteum via transcervical as described by Chapwanya et al. (2010). Tissue samples were immediately snap frozen in liquid nitrogen at −196°C and subsequently at −80°C, until endometrial transcriptome and metabolomics analysis. Estrus records were used to determine on which day of the estrous cycle cows were at the time of biopsy, which averaged day 7 ± 1.2 days (mean±s.d.).

Serum progesterone determination

Serum progesterone was analyzed by a solid-phase radioimmunoassay (RIA) using a commercial kit (MP Biomedicals, Los Angeles, CA, USA) as reported by Ruprechter et al. (2020). The assay sensitivity was 0.11 ng/mL and the intra- and inter-assay coefficient of variation (CV) for Control 1 (0.5 ng/mL) were 19% and 21%, respectively, and for Control 2 (5 ng/mL) 8.9% and 16.6%, respectively.

RNA extraction

The RNA extraction was performed using 1 mL of Trizol and homogenized using an ultra-Turrax T25 homogenizer (Polytron; Brinkmann, Westbury, NY) for 30 s at 6000 rpm. The RNA extraction was performed according to the manufacturer’s instructions, as reported by Fernández-Foren et al. (2023). The concentration and purity of the RNA were determined using a spectrophotometer (NanoDrop ND 1000; Thermo Scientific, Wilmington, DE) and the integrity of the RNA was determined by the Agilent 2100 Bioanalyzer (Agilent Technologies). All samples had an average of A260/280 ratio of 1.95 ± 0.21 and a minimum RNA Integrity Number (RIN) of 4, as required by the sequencing service provider for library generation (Lu et al., 2022). Total RNA was treated with DNase using a DNA-free kit (Ambion, Austin, TX).

Library Generation and RNA Sequencing

RNA samples (n = 16) were sequenced by Novogene Corporation (https://en.novogene.com). Paired-end sequencing libraries with an insert size of about 150 bp were constructed using the RNA-NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). The sequencing was performed using

NovaSeq 6000 (Illumina, Sacramento, CA), with an average read depth of 20 million reads per sample.

RNASeq Analysis: Quality Control, Mapping, and Gene Expression Estimation

The quality of the sequencing reads was assessed using FastQC (Andrews, 2010). When required, adapters and low-quality reads were removed using scythe (v0.991, https://github.com/vsbuffalo/scythe) and sickle (v1.33, https://github.com/najoshi/hoz). A second quality control was performed on the processed reads using FastQC. Clean reads were aligned to the bovine reference genome (Bos taurus, ARS-UCD1.2) using Hisat2 (v2.1.0) (Kim et al., 2015). Hisat2 was run with the parameter '-k 1'; all other parameters were set to default. Finally, for each sample, the total number of reads mapped to each annotated gene in the final assembly was estimated using the featureCounts function from the Rsubread package (Liao et al., 2014).

Sample preparation and proton nuclear magnetic resonance (^{1}H-NMR) spectral acquisition

Endometrial biopsies were homogenized in 300 µL of PBS using an ultra-Turrax T25 homogenizer (Polytron; Brinkmann, Westbury, NY) for 30 s at 6000 rpm. The samples were centrifuged for 10 minutes at 13.8 × g, and the supernatant was collected and stored at -20 °C. Endometrial aqueous extracts were thawed at room temperature, and 200 µL aliquots were mixed with 430 µL of a deuterium oxide buffer solution pD 7.4 and transferred to 5 mm NMR tubes (NE HL5 7, New Era Enterprises Inc., Vineland, NJ, USA) as reported by Dona et al. (2014). Quality control samples were prepared by pooling equal aliquots of all endometrial aqueous extract samples and were analyzed intermittently throughout the analytical sequence. Their spectra were also acquired, and a principal component analysis (PCA) was performed at the end of the run to verify that QC samples clustered tightly at the center of the dataset, indicating analytical stability. All NMR spectra were recorded at 25 °C on a Bruker AVANCE III 500 NMR spectrometer operating at ^{1}H and ^{13}C frequencies of 500.13 and 125.76 MHz, respectively (López-Radcenco et al. 2021). 1D ^{1}H free induction decays were zero-filled to 64 K points and apodized with a 0.3 Hz exponential window function before Fourier transformation. ^{1}H NMR spectra were manually phased and baseline corrected using MNova (version 10.0, MestreLab Research, S.L., Santiago de Compostela, Spain) and referenced to the α-glucose resonance at 5.22 ppm present in all samples. Manually-selected spectral regions were aligned, and the data was normalized to the total spectral area after excluding the residual water resonance signal (4.60–5.00 ppm). The resulting data matrix was then exported as a text file for multivariate analyses.

Metabolite identification and quantification

Metabolites were identified by comparison of ^{1}H NMR data against spectral repositories, including the Biological Magnetic Resonance Bank (BMRB) (Hoch et al., 2023), the Human Metabolome Database (HMDB) (Wishart et al., 2022), and Chenomx (version 9, Chenomx, Inc., Edmonton, Canada). When required, metabolite identification was confirmed with data from 1D-TOCSY and HSQC spectra. Variations in the levels of unambiguously identified metabolites were estimated using relative concentrations. This figure was computed as the ratio between the area from individual metabolite ^{1}H NMR signal and the total area of the spectrum, which was used for subsequent univariate analysis.

Statistical Analyses and Bioinformatics

Serum progesterone

Serum progesterone was analyzed using a generalized linear mixed model (GLIMMIX procedure; SAS Studio®), with fixed effects defined as treatment, parity, days after injection (day) and their interactions. The day of the estrous cycle at the biopsy, determined from the day of estrus (day 0) was used as a covariate. The cow was used as the experimental unit. The covariance structure was autoregressive order 1, as it better fitted the Akaike information criterion (AIC) value. Significance was considered with alpha ≤ 0.05, and a tendency between 0.05 and 0.10. Post hoc comparisons were performed with Tukey–Kramer test.

RNASeq analysis

Differentially expressed genes (DEGs) were identified using the DESeq2 package (version 1.18.1) in R (Love et al., 2014). For this analysis, only genes with at least one read count in a minimum of three samples were included. Genes with an adjusted P-value ≤ 0.05 (Benjamini and Hochberg, 1995) and an absolute $\log_2$ fold change ($|\log_2 FC|$) ≥ 1 were considered differentially expressed between the groups under study. The area under the curve of progesterone and the day of the estrous cycle in which cows were at the moment of endometrial biopsy were used as covariates. Four pairwise comparisons were performed to evaluate the effects of treatment and parity: (i) T0 versus T21 within primiparous cows (T0PP vs T21PP), (ii) T0 versus T21 within multiparous cows (T0MP vs T21MP), (iii) primiparous versus multiparous cows at T0 (T0PP vs T0MP), and (iv) primiparous versus multiparous cows at T21 (T21PP vs T21MP). These contrasts were specified using the contrast argument in the results() function of DESeq2. Functional enrichment analysis of Gene Ontology (GO, Biological Process category) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was conducted using the clusterProfiler package (Yu et al., 2012) in R. Ensembl gene

IDs from each DEG list were converted to Entrez IDs using the org.Bt.eg.db annotation package. GO enrichment was performed with the enrichGO() function using Bos taurus as reference (OrgDb = org.Bt.eg.db), with Benjamini–Hochberg correction for multiple testing (pAdjustMethod = "BH"), and significance thresholds of $p < 0.05$ and $q < 0.1$. KEGG pathway enrichment was conducted using the enrichKEGG() function, specifying "bta" as the organism code.

Metabolomics analysis

Multivariate statistical analyses, including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), were carried out with the PLS_Toolbox package (version 8.5, Eigenvector Research Inc., Manson, WA, USA) implemented for MATLAB (revision 2017b, The MathWorks Inc., Natick, MA, USA). For all models, the data was mean-centered and scaled using a Pareto factor (van den Berg et al., 2006). To account for individual variability in circulating progesterone levels, residualization of the spectral data matrix was performed in MATLAB prior to multivariate analysis. Specifically, each variable (ppm point) was linearly regressed against the individual area under the curve (AUC) of progesterone, and the resulting residuals were used for subsequent modeling. Cross-validation of all OLPS-DA models was achieved using the random subset method, which involved 20 iterations over data split into 8 equally sized parts. Receiver operating characteristic (ROC) curves were plotted, and area under the curve (AUC) values were calculated to ensure the goodness of fit of the resulting models. A permutation test with 50 iterations was also performed to determine the degree of over-fitting and further validate the discriminant analyses (Ni et al., 2008). The results from these validations are provided in Appendix (Figures A2–A3). Since the sample size was not sufficient to allow reliable permutation testing within each parity group, OPLS-DA models were not stratified by parity. Instead, the effect of parity was assessed through univariate analysis of individual metabolite concentrations. Endometrial metabolomics univariate analyses were performed using a generalized linear mixed model (GLIMMIX procedure; SAS Studio®), with fixed effects defined as treatment, parity, and their interaction. The area under the curve of progesterone and the day of the estrous cycle at the biopsy, determined from the day of estrus (day 0) was used as a covariate.

Results and Discussion

Serum progesterone

Serum progesterone was not affected by the treatment or its interactions. The interaction between parity and day was significant as primiparous cows showed higher progesterone concentrations from day 8 onwards, compared to multiparous cows (P < 0.0001, Figure 1). The different progesterone profile between primiparous and multiparous cows is in line with previous findings (Bruinjé et al., 2017), which have been associated with a higher clearance of steroidal hormones due to the higher milk production in multiparous cows (Sangsritavong et al., 2002).

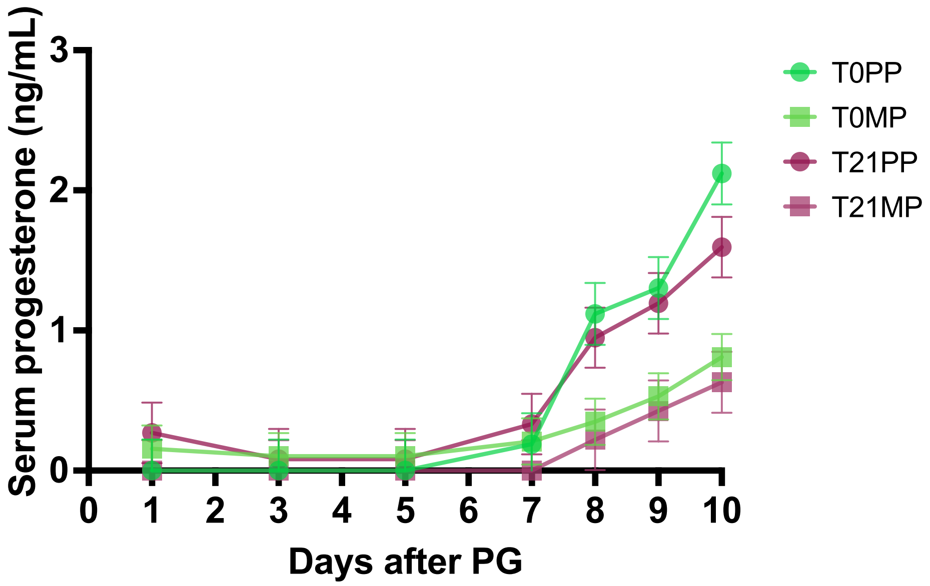


Figure 1. Serum progesterone concentrations following prostaglandin analogue (PG) injection (day 0) until day of endometrial biopsy (day 10), for primiparous (PP, circles) and multiparous (MP, squares) T0 (green, cows grazing with supplementation from calving) and T21 (purple, cows confined with TMR during the first 21 DIM followed by the same management as T0 cows).

RNA-Seq

An average of 53 million reads per sample were obtained. Approximately 92% of the total reads were uniquely mapped to genes annotated in the latest bovine reference genome (ARS-UCD1.2). Of the 27,607 annotated genes in the Bos taurus genome, a total of 21,483 genes passed the pre-filtering criteria and were included in the differential expression analysis. Information on the number of reads, filtering, and alignment per sample is shown in Supplementary table 1. This analysis revealed limited differentiation in the endometrial transcriptome across the contrasts performed (Table 1 and Figure 2). Overall, in multiparous cows, the comparison between T21 and T0 resulted in the identification of only 19 differentially expressed genes (DEGs), while in primiparous cows, 15 DEGs were detected for the same contrast. Despite the use of stringent statistical and biological criteria ($FDR \leq 0.05$, $|\log_2FC| \geq 1$), the limited number of DEGs identified were mostly lowly expressed, often leading to high $\log_2FC$ values; therefore, these results should be interpreted with caution, considering their validation through qPCR. The low level of differentiation in endometrial gene expression is consistent with the lack of differences between treatments in terms of progesterone, one of the key chemical messengers influencing the uterine environment (Forde & Lonergan, 2012; Forde et al., 2012). Moreover, due to the highly regenerative nature of the endometrium (Ohtani et al., 1993; Lara et al., 2018), the small magnitude of differences may be a consequence of how the treatments were applied, as at the time the uterine biopsy collection, the animals had been managed together for approximately 40 days. This time frame could have encompassed one or two estrous cycles, contributing to cellular renewal. However, a basal layer comprising stromal, epithelial, and even stem cells, responsible for its regeneration, persists (Donofrio et al., 2008; Lara et al., 2018), from which we expected to detect some residual effects from the prior management. Indeed, some of the highly expressed genes - and even those with low expression - show biological relevance and concordance with the management the animals were subjected to, as well as to the endocrine-metabolic indicators previously identified. Also, the identified DEGs differed between MP and PP cows' contrasts (Table 1), highlighting a parity-dependent uterine adaptation. Thus, the interpretation of the results described below will be tested by determination of these genes by qPCR.

Table 1. Differentially expressed genes between contrasts of T0 (grazing plus supplementation with PMR from calving) vs T21 (TMR-confinement during the first 21 DIM followed by grazing plus supplementation with PMR) for primiparous (PP) and multiparous (MP)

Contrast	**Ensembl Gene ID**	**Gene Symbol**	**Gene Name**	**Log2Fold Change**	**FDR**
T0MP vs T21MP					
	ENSBTAG00000010299	NOL4	Nucleolar protein 4	27.09	3.92E-05
	ENSBTAG00000014890	CYP19A1	Cytochrome P450 family 19 subfamily A member 1	25.74	0.0001
	ENSBTAG00000043221	SNORA70	Small nucleolar RNA SNORA70	21.83	0.0033
	ENSBTAG00000039722	LOC782061	aldo-keto reductase family 1, member C1-like	19.49	0.0279
	ENSBTAG00000035150	5S_rRNA	5S ribosomal RNA	18.81	0.0199
	ENSBTAG00000052230			18.76	0.0003
	ENSBTAG00000018917	EN2	Engrailed homeobox 2	18.22	0.0001
	ENSBTAG00000014391	CRB2	Crumbs cell polarity complex component 2	15.49	0.0004
	ENSBTAG00000027013	INSM2	INSM transcriptional repressor 2	14.20	1.35E-05
	ENSBTAG00000008182	FOSB	FosB proto-onco, AP-1 transcription factor subunit	7.63	0.0411
	ENSBTAG00000004322	FOS	Fos proto-onco, AP-1 transcription factor subunit	6.88	0.0411
	ENSBTAG00000011178	IL17RB	Interleukin 17 receptor B	2.24	0.0015
	ENSBTAG00000048742	OR52H1D	Olfactory receptor family 52 subfamily H member 1D	-15.22	0.0128
	ENSBTAG00000046742	CLEC2A	C-type lectin domain family 2 member A	-17.17	0.0176
	ENSBTAG00000030343	FGF23	Fibroblast growth factor 23	-17.55	0.0006
	ENSBTAG00000038520	TMPRSS11E	Transmembrane serine protease 11E	-18.96	0.0279
	ENSBTAG00000019764	APOA5	Apolipoprotein A5	-18.97	0.0003
	ENSBTAG00000048344			-19.09	0.0378

ENSBTAG00000038138	ACE3	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 3	-22.09	0.0015
T0PP vs T21 PP				
ENSBTAG00000053589			30.00	1.14E-05
ENSBTAG00000045113	U6	U6 spliceosomal RNA	21.97	0.0167
ENSBTAG00000015210	LOXHD1	Lipoxygenase homology PLAT domains 1	17.99	1.14E-05
ENSBTAG00000051523			17.54	0.0009
ENSBTAG00000045377	SNORA70	Small nucleolar RNA SNORA70	16.73	0.0258
ENSBTAG00000050058			16.17	0.0058
ENSBTAG00000012855	LPL	Lipoprotein lipase	1.83	0.0058
ENSBTAG00000006870	RASGEF1A	RasGEF domain family member 1A	1.17	0.0058
ENSBTAG00000053769	MIR487B	microRNA 487b	-15.36	0.0020
ENSBTAG00000052375	AHRR	Aryl-hydrocarbon receptor repressor	-17.42	0.0211
ENSBTAG00000050353			-18.74	0.0020
ENSBTAG00000052907			-19.60	0.0020
ENSBTAG00000044166	PHOX2B	Paired like homeobox 2B	-21.17	0.0058
ENSBTAG00000048510			-21.57	1.14E-05
ENSBTAG00000054858			-25.06	0.0002

In multiparous cows, 12 genes were upregulated and 7 were downregulated in the T0 group compared to T21 (Table 1). The DEGs with the highest expression levels were *IL17RB*, *FOS*, and *FOSB*, all of which were upregulated in T0 compared to T21 multiparous cows. The *IL17RB* gene encodes a cytokine (IL17) receptor that mediates the Th2-type immune response (Rickel et al., 2008), known to exert important anti-inflammatory roles required for pregnancy establishment (Talukder et al., 2020). When interleukins (IL-17B and IL-17E, also known as IL-25) bind to this receptor, an intracellular signaling cascade is activated, ultimately leading to the activation of the transcription factor NF-κB (Wong et al., 2005). This signaling pathway plays important roles in endometrial receptivity during the early days of the estrous cycle and pregnancy (Ross et al., 2010), and IL-17/IL17RB signaling contributes to embryonic development (Bie et al., 2017). These pathways, including the *IL17RB* gene, were found to be enriched in the endometrium of beef heifers supplemented with omega-3 fatty acids (Waters et al., 2014). These polyunsaturated fatty acids are more abundant in diets based on fresh pasture compared to those containing conserved forages (NRC, 2001; Krusinski et al., 2022), and thus, the difference in *IL17RB* expression could reflect a residual effect of the diet received during the early postpartum period and/or more intake from pastures in T0 MP cows. *FOS* is known to be highly up-regulated in pregnant bovine endometrium, and has been related to cell proliferation and differentiation, immune function, endocrine activity and endometrial health (Adhikari et al., 2022). *FOSB*, a member of the same gene family, also plays a role in cell proliferation and differentiation, although it is less characterized in cattle. Although expressed at low levels, *CYP19A1*—the gene encoding the enzyme responsible for converting androgens into estrogens—was also found to be overexpressed in T0 multiparous cows compared to T21. Since 17β-estradiol is known to induce *FOS* transcription (Girard et al., 2015), this would be consistent with the higher expression of *FOS* and *FOSB* observed in multiparous cows at T0. While this enzyme is primarily active in the ovary, there is solid evidence of its expression in the human endometrium, enabling intracrine production of estradiol (Gibson et al., 2018). In an elegant study, it was demonstrated that aromatase activity in mouse uterine stromal cells undergoing decidualization was essential for the formation of a receptive endometrium capable of supporting a viable pregnancy (Das et al., 2009). However, its expression in the bovine endometrium remains poorly characterized. On the other hand, there is evidence that negative energy balance suppresses ovarian *CYP19A1* expression through increased inhibin and decreased adiponectin levels (Rak et al., 2017). Specifically, Girard et al. (2015) predicted inhibition of β-estradiol signaling in cows with elevated BHB, which is consistent with the low expression observed in multiparous T21 cows, who exhibited higher BHB levels after the change from confinement to grazing (22-60 DIM, Mendina et al. submitted). Additionally, although expressed at low levels, ENSBTAG00000039722 was found to be upregulated in multiparous T0 cows compared to T21. This transcript has not been characterized in cattle, but it shares a high sequence similarity (close to 100%) with the gene encoding prostaglandin F synthase 1 (PGFS) in bison and other species. Despite the low expression, its higher abundance in multiparous T0 compared to T21, in combination with other genes, may have biological relevance. Endometrial prostaglandin production in cows can be stimulated by estradiol (Li et al., 2020), in line with the also upregulated aromatase, and is also regulated by the NF-κB signaling pathway (Waters et al., 2014), which aligns with the higher expression of *IL17RB*. Prostaglandins play a fundamental role in the early days of the estrous cycle and in the preparation for early pregnancy, regulating conceptus elongation and mediators of endometrial responses to progesterone and IFNT in the ovine uterus (Dorniak et al., 2011). Additionally, higher concentrations of prostaglandins have been found in the uterine lumen during early pregnancy in heifers classified as highly fertile compared to subfertile animals (Moraes et al., 2020). Other upregulated genes in T0 cows, such as *CRB2* and *EN2*, are implicated in embryonic development, while *SNORA70* is predicted to participate in rRNA modification within the nucleolus. Although its role in cattle has not been fully elucidated, *SNORA70* has been associated with sexual precocity in Bos indicus (Melo et al., 2019).

Downregulated genes in T0 multiparous cows included *APOA5, TMPRSS11E, FGF23, CLEC2A, and OR52H1D*. Although to be considered with caution due to its low expression level, *APOA5* encodes apolipoprotein A5, a key regulator of triglyceride metabolism inversely related to plasma TAG concentrations (Garelnabi et al., 2013). The underlying mechanisms remain unclear, but increased APOA5 expression has been linked to increased VLDL turnover (Fruchart-Najib et al., 2004) via lipoprotein lipase activation (Merkel et al., 2005), which may facilitate TAG utilization by tissues (Drackley, 1999). Therefore, its higher expression in endometrium of T21 cows at 60 DIM may reflect a greater energy demand and mobilization at that time (Khan et al., 2014), consistent with previous findings of the metabolic adaptation of T21 cows to grazing (Mendina et al. submitted). Additional downregulated transcripts in T0 multiparous cows included *FGF23*, Fibroblast Growth Factor 23, a phosphaturic hormone that suppresses calcitriol synthesis (Takashi and Fukumoto, 2018). The up-regulation in T21 cows is consistent with findings that calcitriol was also an inhibited upstream regulator in high-BHB cows (Girard et al., 2015). However, endometrial expression of FGF23 could play a role in paracrine signaling, potentially influencing nutrient transporter expression and histotroph secretion (Stenhouse et al., 2023), being beneficial during pregnancy establishment (Stenhouse et al., 2022). Nonetheless, its role in its stage of estrous cycle in the bovine endometrium remain to be elucidated. On the other hand, the down-regulation of *CLEC2A*, a gene promoter of the innate immune response, may also be beneficial for reproductive success given the important role of immune modulation in pregnancy establishment. However, the role of CLEC2A in ruminant reproduction is still poorly characterized. Taken together, the profile of expression of this group of genes in T0 multiparous cows seems to contribute to a favorable uterine milieu for an eventual pregnancy. Whether these differences result from the metabolic stress experienced by T21 cows due to the management change, or stem from residual effects of the differential feeding received in the early postpartum days, requires further investigation.

In primiparous cows, 8 genes were upregulated and 7 were downregulated in the T0 group compared to T21. Notably, *LPL* (lipoprotein lipase) and *LOXHD1* (lipoxygenase homology PLAT domains 1) were significantly upregulated in the T0 group. *LPL* encodes a key enzyme responsible for the hydrolysis of circulating triglycerides, potentially enhancing energy availability to endometrial tissue or contributing with triacylglycerols to the uterine histotroph, which may serve as an energy source for the developing embryo at this stage (Forde et al., 2010; Araújo et al., 2016). Its lower expression in T21 primiparous cows raises the question of whether they might be facing some difficulty in incorporating these substrates into the uterus, or whether they might be relying on alternative energy sources at the time of sample collection. LOXHD1 contains lipoxygenase-like domains, and although its function in cattle remains unclear, studies in mice indicate that this gene is essential for normal cell function. Additionally, RASGEF1A, upregulated in T0 cows, encodes a guanine nucleotide exchange factor linked to cell migration and Ras-mediated signaling, a crucial signaling pathway in cells, primarily responsible for regulating cell growth, proliferation, differentiation, and survival. However, AHRR (aryl hydrocarbon receptor repressor) was downregulated in T0 cows, and this gene plays roles in cell growth and differentiation, involved in pathways such as white adipocyte differentiation and cytochrome P450-mediated metabolism of fatty acids and steroid hormones. Two non-coding RNAs -U6 (a small nuclear RNA) and SNORA70- were among the most highly upregulated transcripts in T0 primiparous cows (Table 1). U6 is a highly conserved spliceosomal RNA essential for pre-mRNA processing, whereas the upregulation of SNORA70, as in T0 multipaorus cows, reinforces its potential relevance in RNA processing and reproductive function. Among the downregulated genes in primiparous cows, three non-coding RNAs -MIR487B (microRNA), ENSBTAG00000052907 (a putative lncRNA), and ENSBTAG00000054858 (a putative miRNA)- are thought to be involved in transcriptional and post-transcriptional regulation. Previous findings revealed higher betaine, dimethylglycine, and formate in serum metabolomics in T0 primiparous cows at 21 DIM, which are related to the one-carbon metabolism pathway (Mendina et al. submitted). It is possible that the pronounced nutritional deficiency in this growing category during early postpartum could have repercussions on methylneogenesis (Custodio et al., 2017; McFadden et al., 2020; Arshad and Santos, 2024), potentially affecting uterine methylation processes, with consequences for reproductive success (Walker and Mitchell, 2013). Further research in this field is needed to understand the interplay between these mechanisms.

The contrasts between primiparous and multiparous cows within treatments revealed 19 DEGS for T0 and 12 DEGs for T21 cows (Table 2). Interestingly, 4 DEGs were common for both contrasts, as primiparous cows showed an up-regulation of SLC27A6, which encodes a protein involved in the uptake of long-chain fatty acids into the cell. The expression of SLC27A6 in endometrium is positively associated with P4 concentrations (Forde et al., 2012), which is in agreement with the differences in concentration of this hormone between primiparous and multiparous cows in the present study. Forde et al. (2009) attributed to SLC27A6 the capacity to enrich the histotroph with fatty acids to sustain early embryo developmental stages. However, Moraes et al. (2018) found an endometrial up-regulation of SLC27A6 in infertile compared to subfertile or fertile nonpregnant heifers, which could indicate a greater challenge for primiparous cows in sustaining a pregnancy at the time of sample collection. Among down-regulated genes were R3HDML, NPBWR1 and a novel gene (ENSBTAG00000054653). R3HDML is required for skeletal muscle development and regeneration (Sakamoto et al., 2019) and adipogenesis in cattle (Wang et al., 2025), and have been positively associated with IGF-1 in mice (Sakamoto et al., 2019). NPBWR1 encodes a peptide receptor with neuroendocrine function implicated in regulation of feeding behavior and energy homeostasis (Sakurai, 2013). Little is known about ENSBTAG00000054653, but recently, it has been identified among candidate genes associated with uterine health in primiparous Holstein cows (Zhang et al., 2024). Altogether, the upregulation of pathways related to increased fatty acids uptake as an energy source, while having an inhibition of genes related to anabolism, energy homeostasis and utetine health, could reflect the metabolic and physiological challenges faced by primiparous cows during their first lactation, where the simultaneous demands of growth and milk production, may compromise energy balance, potentially affecting fertility.

Table 2. Differentially expressed genes between contrasts of primiparous (PP) and multiparous (MP) within treatment T0 (grazing plus supplementation with PMR from calving) or T21 (TMR-confinement during the first 21 DIM followed by grazing plus supplementation with PMR)

Contrast	Ensembl Gene ID	Gene Symbol	Gene Name	log2FoldChange	FDR
T0PP vs T0MP					
	ENSBTAG00000053589			22.90	0.0077
	ENSBTAG00000010385	LOC523130		22.58	0.0104
	ENSBTAG00000014234	CCDC196	Coiled-coil domain containing 196	22.16	0.0057
	ENSBTAG00000048742	OR52H1D	Olfactory receptor family 52 subfamily H member 1D	21.66	6.61E-06
	ENSBTAG00000039065	HS6ST3	Heparan sulfate 6-O-sulfotransferase 3	20.31	0.0479
	ENSBTAG00000016293	TRIM77	Tripartite motif containing 7	19.95	0.0113
	ENSBTAG00000046742	CLEC2A	C-type lectin domain family 2 member A	18.45	0.0157
	ENSBTAG00000030343	FGF23	Fibroblast growth factor 23	18.23	0.0026
	ENSBTAG00000006732	NRCAM	Neuronal cell adhesion molecule	2.35	0.0442
	ENSBTAG00000034145			1.88	0.0280
	ENSBTAG00000004860	SLC27A6	Solute carrier family 27 member 6	1.88	0.0026
	ENSBTAG00000006870	RASGEF1A	RasGEF domain family member 1A	1.06	0.0280
	ENSBTAG00000009085	SLC35A5	Solute carrier family 35 member A5	1.04	7.40E-07
	ENSBTAG00000048510			-14.64	0.0479
	ENSBTAG00000053769	MIR487B	MicroRNA 487b	-15.13	0.0043
	ENSBTAG00000013220	R3HDML	R3H domain containing like	-20.38	0.0479
	ENSBTAG00000016159	NPBWR1	Neuropeptides B and W receptor 1	-21.04	0.0284
	ENSBTAG00000054653			-21.68	0.0193

	ENSBTAG00000045847	CTXN2	Cortexin 2	-22.04	0.0052
T21PP vs T21MP					
	ENSBTAG00000010299	NOL4	Nucleolar protein 4	35.58	0.0001
	ENSBTAG00000018917	EN2	Engrailed homeobox 2	19.68	0.0290
	ENSBTAG00000014391	CRB2	Crumbs cell polarity complex component 2	17.51	0.0290
	ENSBTAG00000027013	INSM2	INSM transcriptional repressor 2	17.25	2.76E-05
	ENSBTAG00000004860	SLC27A6	Solute carrier family 27 member 6	2.20	0.025431
	ENSBTAG00000015210	LOXDH1	Lipoxygenase homology PLAT domains 1	-16.25	0.0434
	ENSBTAG00000045377	SNORA70	Small nucleolar RNA SNORA70	-22.53	0.0290
	ENSBTAG00000045113	U6	U6 spliceosomal RNA	-33.89	0.0009
	ENSBTAG00000013220	R3HDML	R3H domain containing like	-41.07	2.40E-06
	ENSBTAG00000054653			-44.12	1.47E-07
	ENSBTAG00000049031	OR12D2G	Olfactory receptor family 12 subfamily D member 2G	-44.63	1.21E-07
	ENSBTAG00000016159	NPBWR1	Neuropeptides B and W receptor 1	-48.50	3.29E-09

In T21 parity contrast, four DEGs were also common for the multiparous contrast between treatments. INSM2, which was downregulated in T21 multiparous cows compared primiparous T21, regulates glucose-stimulated insulin secretion (Wang et al., 2018), which is in line with the high BHB levels of multiparous T21 at this time. Also, the downregulation of CRB2 and EN2 in multiparous T21, as mentioned before, could be detrimental for embryonic development.

In the contrast between primiparous and multiparous T0 cows, CCDC196 was up-regulated in primiparous. This gene has been recently related to fertility traits in cattle such as the increase in calving interval, being up-regulated in low-fertility cows (van den Berg et al., 2024). Other up-regulated genes in primiparous T0 compared to multiparous T0 cows were TRIM77, which is predicted to be involved in innate immune response and regulate gene expression; SLC35A5, suggested to be involved in glycosylation and nucleotide sugar transport into the Golgi apparatus (Kamiyama and Sone, 2024); NRCAM, involved in signal transduction pathways regulating cell differentiation, proliferation, or migration (McClure et al., 2013), and HS6ST3, implicated in proliferation and differentiation, adhesion, migration, inflammation, and other diverse processes (Jiang et al., 2011). Overall, the upregulation of CCDC196, TRIM77, SLC35A5, NRCAM, and HS6ST3 in the uterus of primiparous cows compared to multiparous in T0, may reflect a distinct uterine microenvironment and could be a consequence of different progesterone concentrations or physiological demands. Although many of the up-regulated genes in primiparous are related to tissue remodeling, immune signaling, and cell–cell communication, possibly indicating an enhanced endometrium milieu that could provide a better capacity to sustain an eventual pregnancy compared to multiparous (Santos et al., 2009), further investigation is needed.

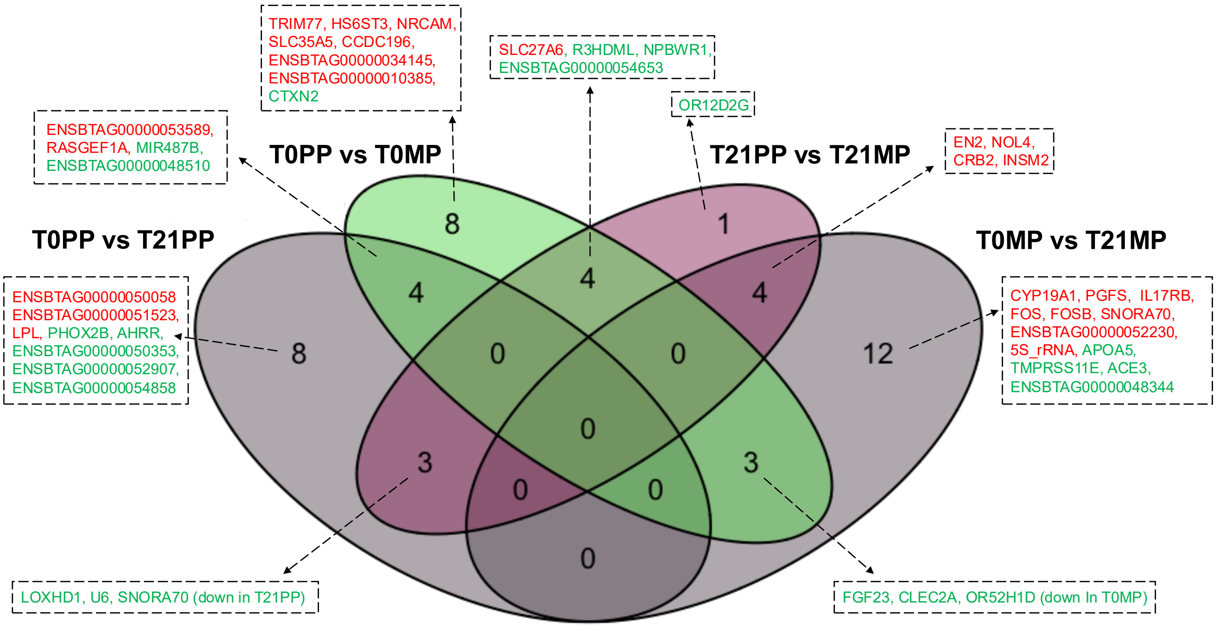


Figure 2. Venn diagram showing the differentially expressed genes in the treatment contrasts (grey circles) for primiparous cows (T0PP vs T21PP) and multiparous cows (T0MP vs T21MP), or between parities within the T0 (green, T0PP vs T0MP) or T21 (purple, T21PP vs T21MP) treatments, at 60 days in milk and day 7 of the estrous cycle. Up-regulated (red) or down-regulated (green) genes refer to the first group mentioned in the name of each contrast, unless otherwise indicated (genes shared between multiple contrasts are listed at the bottom of the figure).

Endometrial metabolomics

To explore potential clustering patterns based on treatment and parity, a principal component analysis (PCA) was performed as an unsupervised classification method using the data obtained from the ^{1}H NMR of both endometrial and serum samples. In the case of endometrial metabolomics, PCA did not reveal a clear separation between treatments or a visible clustering by parity (Fig. 3). After conducting a supervised classification (OPLS-DA), some separation between treatments was observed but the model performance was suboptimal (AUC = 0.68). Moreover, it failed to pass permutation test validation, suggesting that the overall metabolomic profile did not provide sufficient discriminatory power to distinguish between treatments.

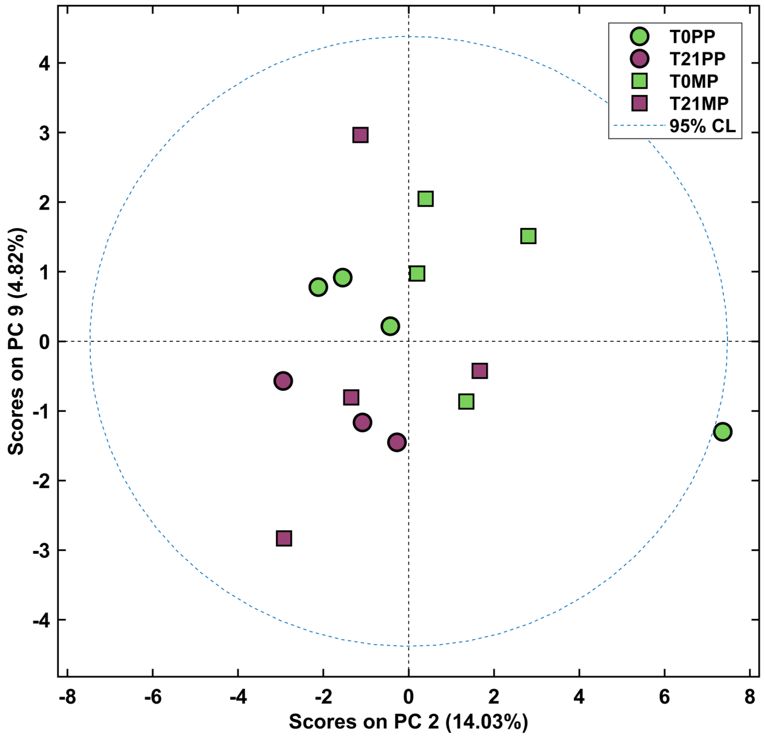


Figure 3. PCA score plot obtained from ^{1}H-NMR spectral data of endometrial samples from primiparous (circles) and multiparous (squares) T0 (green) and T21 (purple) at 60 DIM.

In contrast, the univariate analysis revealed specific differences that the multivariate approach failed to detect. Carnitine concentration showed a tendency to be influenced by both treatment (P = 0.071) and parity (P = 0.061), with higher levels observed in T21 versus T0 cows and in multiparous versus primiparous cows. Carnitine plays a key role in the transport of long-chain fatty acids into mitochondria for β-oxidation and has also been reported to enhance gluconeogenesis from amino acids such as alanine (Carlson et al., 2007). In line with the higher APOA5 endometrial expression found in T21 multiparous cows, this finding could indicate a higher energy demand in this group compared to T0 multiparous cows. Moreover, dietary carnitine supplementation has been shown to increase plasma BHB concentrations due to enhanced β-oxidation, without affecting circulating NEFA levels (Carlson et al., 2007). Although no differences in endometrial BHB were found, multiparous T21 cows exhibited a serum BHB profile consistently elevated throughout the post-transition period, compared to their T0 counterparts. The relative concentration of lactate was significantly affected by treatment ($P = 0.012$), with T0 cows showing higher levels than T21 cows. Additionally, there was a tendency for the interaction between treatment and parity ($P = 0.10$), as lactate levels were significantly higher in T0 multiparous cows compared to their counterparts in T21 ($P = 0.03$; Figure 4A). Conversely, creatine levels tended to be higher in T21 cows ($P = 0.089$). These findings could also indicate a derivation of lactate as a substrate for glucose or energy supply in the endometrium (Chen and Dean, 2023) , as well as an increased catabolism of body proteins resulting in higher creatine (Sadri et al., 2023). Isoleucine concentration was affected by the interaction between treatment and parity, as primiparous T21 showed higher concentrations than primiparous T0 and multiparous T21 cows ($P < 0.05$). In contrast, acetate concentrations were significantly greater in the endometrium of T21 cows compared to T0 cows ($P = 0.033$).

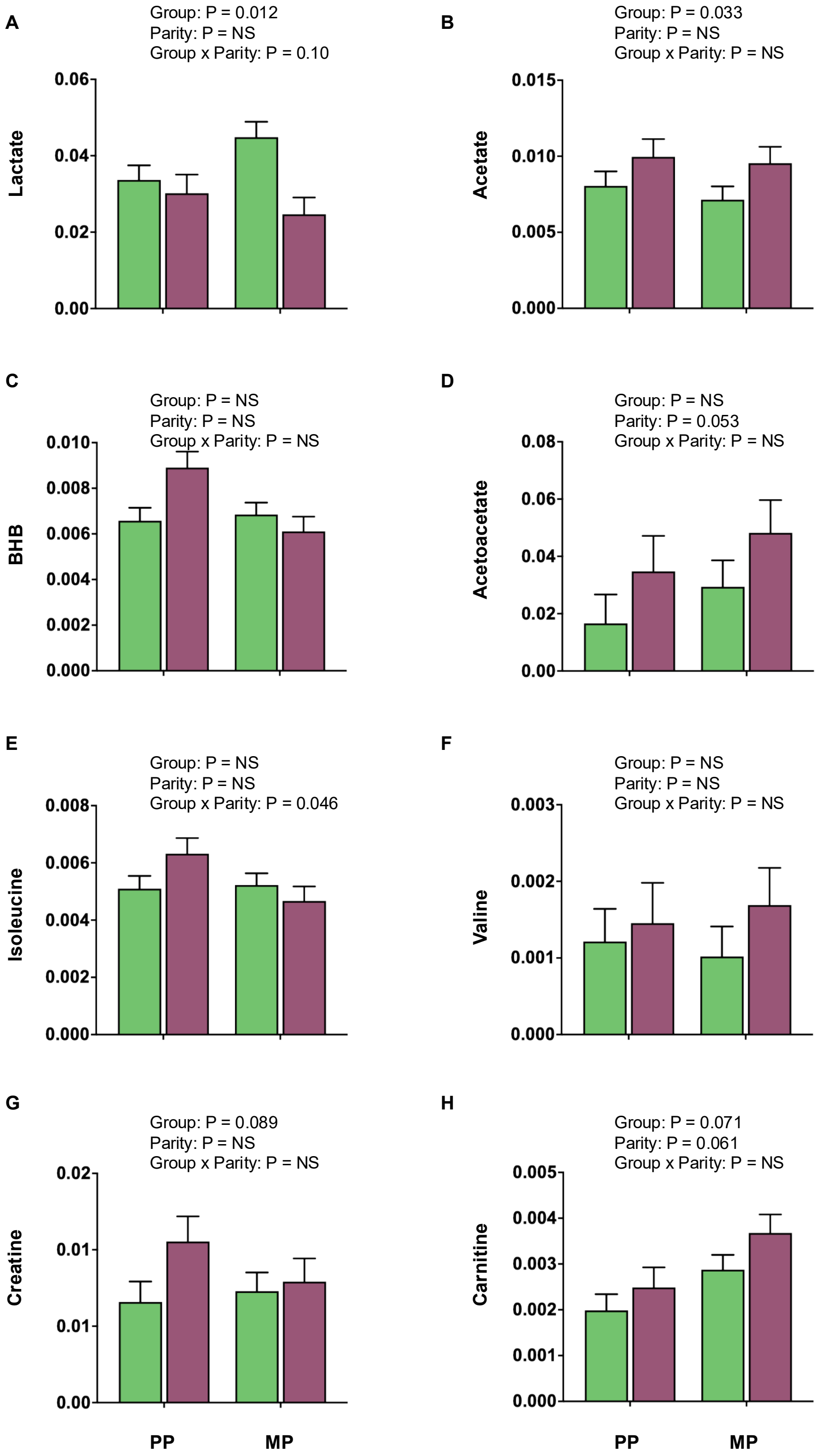


Figure 4. Relative concentrations of endometrial metabolites in primiparous (PP) and multiparous (MP) of T0 (green) and T21 (purple) groups at 60 DIM. Data are presented as mean ± SEM.

Taken together, these findings support the notion that T21 cows -despite showing initial benefits in terms of energy balance during the early postpartum period- experienced a later metabolic adjustment following the transition to pasture. This metabolic adaptation is further explored in Mendina et al. (submitted), where changes in serum metabolites and productive performance are described in detail. Notably, signs of this adaptation were still evident 40 days after the shift to a pasture-based management, as reflected in both systemic metabolites and local (endometrial) transcriptomic and metabolomics. These delayed effects underscore the importance of considering both short- and long-term consequences of dietary management strategies on reproductive tract physiology and overall metabolic health.

Data and model availability statement

Notes

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Author ORCIDs

G.R. Mendina: https://orcid.org/0000-0003-1311-4421

E. Jara: https://orcid.org/0000-0002-9750-2642

V. de Brun: http://orcid.org/0000-0003-2133-6075

M.L. Adrien: https://orcid.org/0000-0001-5914-1981

C. Rivoir: https://orcid.org/0000-0001-8269-0815

P. Chilibroste: https://orcid.org/0000-0001-9579-9967

G. Moyna: https://orcid.org/0000-0002-0727-1079

A. López: https://orcid.org/0000-0003-0203-0732

A. Meikle: http://orcid.org/0000-0002-0847-8629

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Supplementary Table 1. Sequencing statistics from RNA samples of T0 and T21 primiparous and multiparous cows at day 7 of the estrous cycle and 60 DIM

Sample	raw_reads	clean_reads	mapped_reads	Q30_reads	% alignment
U2372T0	60371322	58923840	56646384	55125884	96.13
U2388T0	52041120	50757048	48771523	47414166	96.09
U4004T0	54726066	53535818	51307200	50032980	95.84
U4006T0	44256332	43252320	41509323	40549379	95.97
U2391T21	53227906	51937272	49796300	48545691	95.88
U2392T21	62111746	60628588	58551005	57060676	96.57
U4010T21	43860544	42562604	41137766	39876173	96.65
U804T0	65339342	63843972	61418973	59810776	96.20
U2680T0	48125172	46964514	45120904	43938646	96.07
U2690T0	51327432	50210858	48273143	47015688	96.14
U3748T0	56092112	54578970	52450431	50864866	96.10
U6222T0	43273792	42331638	40229353	39236304	95.03
U401T21	50794444	49561296	47871072	46726643	96.59
U813T21	42960442	41978476	40188161	38878654	95.74
U814T21	71986088	70239734	66236231	63714108	94.30
U3612T21	47185322	46041612	43654850	42291129	94.82

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Increased expression of interferon-stimulated gene 15 (ISG15) in cervical cells on day 14 of pregnancy in Holstein heifers. **Mendina GR**, de Brun V, Adrien ML, Pons V, Vivian Paradizo R, Gil J, Rocha CC, Binelli M, Meikle A. (2025) JDS Communications. 6:165–170. doi: 10.3168/jdsc.2024-0596.

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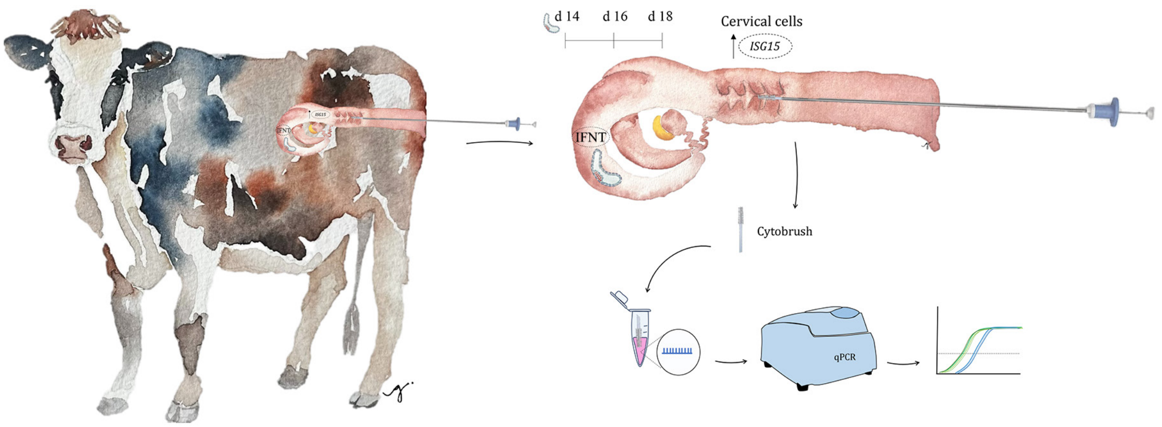
Short Communication
Physiology

Increased expression of interferon-stimulated gene 15 (*ISG15*) in cervical cells on day 14 of pregnancy in Holstein heifers

Graciana R. Mendina,[1]* Victoria de Brun,[2] Maria de Lourdes Adrien,[1] Victoria Pons,[1] Rodrigo Vivian Paradizo,[3] Jorge Gil,[1] Cecilia C. Rocha,[4] Mario Binelli,[4] and Ana Meikle[2]

Graphical Abstract

Increased cervical *ISG15* mRNA expression from day 14 to 18 of pregnancy in Holstein heifers

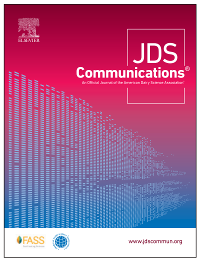


Summary

In cattle, the ability to diagnose pregnancy status before the natural return to estrus allows for the detection of early embryo losses and the use of resynchronization programs for rebreeding in a short period of time. The production of interferon tau (IFNT) by trophoblastic cells starts in the early embryo stages. Expression of IFNT-stimulated genes in the cervix has been reported as an early pregnancy diagnostic tool as early as day 17 of pregnancy. We compared the expression of *ISG15* in cervical cells between pregnant and cyclic heifers (control, sham-inseminated) on days 14, 16, and 18 after insemination. The expression levels of *ISG15* in cervical cells were significantly greater in pregnant compared with control heifers on day 14, and remained greater on days 16 and 18. A receiver operator characteristic (ROC) curve analysis showed the most accurate prediction of pregnancy on day 16.

Highlights

- Pregnant heifers had greater *ISG15* expression in cervical cells as early as day 14 compared with controls.
- Cervical *ISG15* expression was greater on day 16 than on day 14 in pregnant heifers.
- A ROC curve analysis showed the most accurate prediction of pregnancy on day 16.



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[1] Departamento de Ciencias Veterinarias y Agrarias, Facultad de Veterinaria, Universidad de la República, Paysandú 60000, Uruguay, [2] Departamento de Clínicas y Hospital Veterinario, Facultad de Veterinaria, Universidad de la República, Montevideo 18000, Uruguay, [3] Departamento de Producción Animal y Pasturas, Facultad de Agronomía, Universidad de la República, Paysandú 60000, Uruguay, [4] Department of Animal Sciences, University of Florida, Gainesville, FL 32611. *Corresponding author: g.rmendina@gmail.com. Received April 15, 2024. Accepted September 15, 2024.

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Short Communication
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Increased expression of interferon-stimulated gene 15 (*ISG15*) in cervical cells on day 14 of pregnancy in Holstein heifers

Graciana R. Mendina,[1]* Victoria de Brun,[2] Maria de Lourdes Adrien,[1] Victoria Pons,[1]
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Abstract: In cattle, expression of IFN-stimulated genes in the female reproductive tract has been reported as an early pregnancy diagnostic tool, as early as d 17 of pregnancy. The hypothesis of this study was that expression of *ISG15* in the cervix of pregnant heifers is increased on d 14 of pregnancy. The objective was to compare the expression of *ISG15* in cervical cells between pregnant and cyclic heifers (control, sham-inseminated) on d 14, 16, and 18 after insemination (d 0). Holstein heifers were submitted to an estrus synchronization protocol and inseminated with extender only ("control," n = 6), or with regular semen (n = 15). Heifers were classified as pregnant (n = 10) by ultrasound at d 30 through the detection of a viable embryo with a heartbeat. Blood samples from the coccygeal vein were collected to determine serum progesterone concentrations on d 14, 16, and 18. The expression of *ISG15* and *PGR* in cervical cells collected through cytobrush was measured on d 14, 16, and 18. A receiver operating characteristic (ROC) curve was calculated to quantify the pregnancy diagnostic accuracy of *ISG15* and *PGR* expression. The expression levels of *ISG15* in cervical cells were significantly greater in pregnant compared with control heifers on d 14, and remained greater on d 16 and 18, whereas differences in PGR were observed only on d 18. Scatter plots and ROC analyses showed the most accurate prediction of pregnancy for *ISG15* on d 16. In conclusion, cervical cells express greater *ISG15* mRNA in pregnant versus control heifers as early as d 14 postinsemination, with the best accuracy on d 16.

The ability to diagnose the pregnancy status before the natural return to estrus allows the detection of early embryo losses and the use of resynchronization programs for rebreeding in a short period (Motta et al., 2020). The capacity to reduce the time to conception and improve reproductive efficiency increases the profitability of dairy (Inchaisri et al., 2010) and beef (Lamb and Mercadante, 2016) cattle production systems. However, the search for an early pregnancy biomarker has yielded limited results.

To achieve a successful pregnancy, the semi-allogeneic conceptus produces a trophoblast-derived cytokine called interferon tau (**IFNT**) to induce immunological tolerance toward itself and prevent luteolysis (reviewed by Forde and Lonergan, 2017; Talukder et al., 2020). The production of the IFNT starts as early as d 7 (Sponchiado et al., 2017; Rashid et al., 2018), reaching a peak in its expression on d 15 to 16 of pregnancy (Farin et al., 1990). Since the discovery that IFNT triggers the expression of interferon-stimulated genes (**ISG**) in the bovine endometrium (Naivar et al., 1995), many researchers sought to use them as early pregnancy markers in a variety of tissues and cell types.

Greater expression of ISG has been found in pregnant cows' white blood cells mostly between d 16 and 20 (Gifford et al., 2007; Pugliesi et al., 2014; Haq et al., 2016; Sheikh et al., 2018; Melo et al., 2020b), liver on d 18 (Meyerholz et al., 2016), corpus luteum on d 16 (Yang et al., 2010), endometrium from d 15 (Austin et al., 2004; Forde et al., 2011; Moraes et al., 2020; Adhikari et al., 2022), and cervical and vaginal cells from d 17 to 18 (Kunii et al., 2018; Ferraz et al., 2021; Domingues et al., 2024). Moreover, greater *ISG15* mRNA expression was detected as early as d 7 postinsemination only in the uterotubal junction of the uterine horn ipsilateral to the CL of pregnant cows, but not in other regions of the uterine horns, suggesting that at this early stage of development, closer proximity with the embryo is necessary to stimulate the expression of ISG (Sponchiado et al., 2017). However, sampling the cranial endometrium to measure ISG expression as an early biomarker of pregnancy is impracticable to the maintenance of the pregnancy.

Recently, researchers found increased expression of *ISG15* as early as d 17 in cervical and vaginal cells of lactating Holstein cows (Kunii et al., 2018; Domingues et al., 2024). Interestingly, the cervical expression of *ISG15* was around 16-fold greater than the traditional methodology of measurement in blood immune cells (Kunii et al., 2018; Domingues et al., 2024). Nevertheless, in these studies, *ISG15* was detected in pregnant versus nonpregnant cows, without comparing to a cyclic noninseminated group, which is necessary to establish a basal threshold of *ISG15* expression for pregnancy diagnosis.

It is well known that progesterone (**P4**) modifies the reproductive tract physiology for pregnancy maintenance. Although it is accepted that in ruminants, in vivo implantation events are preceded by the loss of expression of progesterone receptor (*PGR*) in the luminal epithelium (Bazer et al., 2008), it has been also shown in

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[1]Departamento de Ciencias Veterinarias y Agrarias, Facultad de Veterinaria, Universidad de la República, Paysandú 60000, Uruguay, [2]Departamento de Clínicas y Hospital Veterinario, Facultad de Veterinaria, Universidad de la República, Montevideo 18000, Uruguay, [3]Departamento de Producción Animal y Pasturas, Facultad de Agronomía, Universidad de la República, Paysandú 60000, Uruguay, [4]Department of Animal Sciences, University of Florida, Gainesville, FL 32611. *Corresponding author: g.rmendina@gmail.com. Received April 15, 2024. Accepted September 15, 2024.

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in vitro bovine endometrial cells that IFNT induces the expression of *PGR* and that there are IFN genomic binding sites for IFN-responsive factors in the *PGR* gene (Palma-Vera and Einspanier, 2016). Overall, less information is available on *PGR* in the cervix, and we have not found reports on cervical *PGR* expression during early pregnancy.

Thus, as there is evidence that IFNT is produced even before the maternal recognition of pregnancy (i.e., d 16) and that it induces the expression of ISG in surrounding reproductive tissues, we hypothesize that pregnant heifers have greater expression of *ISG15* in cervical cells as early as d 14 postinsemination compared with control sham-inseminated heifers. The aim of this study was to compare the gene expression of *ISG15* and *PGR* on d 14, 16, and 18 postinsemination in the cervix of pregnant and control Holstein heifers.

Cyclic Holstein heifers between 15 and 18 mo of age and weighing 360 ± 36 kg, maintained at the Experimental Station Dr. Mario A. Cassinoni, Facultad de Agronomía, Universidad de la República, Paysandú, Uruguay (32° 23′07.6″ S 58° 03′17.9″ W), were used for this study. The research protocol was approved by the Ethics Committee of Universidad de la República, CEUA-CHEA ID 14/2023-Exp. 311170–000129–23). The experiment was performed from October to November of 2023. Heifers were kept in grazing paddocks (*Lolium multiflorum* and *Trifolium pratense*), and received supplementation (1.6 and 0.8 kg/animal per day of soybean hulls and ground corn, respectively) and water ad libitum. Heifers were submitted to an estrus synchronization protocol based on 2 injections of 2 mL of $PGF_{2\alpha}$ analog ($PGF_{2\alpha}$; D-cloprostenol, 0.075 mg/mL, Ciclar, Zoovet, Argentina), i.m., 11 d apart. In both injections, heifers were fitted with estrus detection patches (Estrotect, Rockway Inc., USA). Visual estrus detection was conducted 3 times a day by a single experienced operator, starting 12 h after the second $PGF_{2\alpha}$ injection, and continued for 96 h. Heifers were considered in estrus when standing to be mounted or when more than 50% of the silver coating of the patch was removed. Heifers were distributed randomly in 2 treatments: controls, sham-inseminated with semen extender only (n = 6), and inseminated with regular semen (n = 15) from a single Holstein bull previously used in the experimental station herd with satisfactory results. A pool of 2 straws of the same batch used in the experiment was analyzed for kinematics by the computer-assisted semen analyzer (CASA) system, as well as subsequent morphological evaluation. Animals were inseminated between 6 and 12 h after the first visualized standing estrus event or patch indication. Blood samples were taken from the coccygeal vein using an evacuated tube system (Vacuette 8 mL Serum Beads Clot Activator, Greiner Bio-One GmbH) on d 14, 16, and 18 after insemination. Blood samples were centrifuged at 1,680 × *g* for 10 min at room temperature and serum was stored at −20°C until further analysis. Epithelial cervical cell samples were obtained by a single experienced operator, blinded to group assignment, using a cytological brush (Cytobrush, Sakira S.A., China) coupled to the tip of a conventional AI gun, covered by a disposable AI sheath and protected by a sanitary sheath, as described previously by Cardoso et al. (2017), on d 14, 16, and 18 after insemination. The apparatus was inserted via the cervix and rotated to harvest cells from the cervical canal near the external os of the cervix. The cytobrush was uncoupled from the apparatus and placed into a 2-mL cryotube filled with 1 mL of Trizol reagent (Life Technologies) for mRNA extraction and stored immediately in liquid nitrogen at −196°C. Samples were then stored at −80°C until mRNA extraction. Confirmatory pregnancy diagnosis was performed on d 30 postinsemination using transrectal ultrasonography with a linear transducer (Aloka 500, 7.5 MHz, Tokyo, Japan) through the detection of a viable embryo with a heartbeat. Animals were then classified as pregnant (n = 10) and nonpregnant. Nonpregnant heifers were excluded from the analysis because of the uncertain outcome of their pregnancies, which may have included embryo loss at different stages, associated with transient increases in IFNT of varying magnitudes. A subsequent diagnosis was performed on d 90, confirming that 4 heifers did not maintain pregnancy after d 30; however, due the limited number of animals no further analysis was performed other than control versus pregnant heifers on d 30. Serum P4 was determined on d 14, 16, and 18 postinsemination by a solid-phase RIA using a commercial kit (MP Biomedicals, Los Angeles, CA) as reported by Ruprechter et al. (2020). All samples were analyzed in a single assay; the sensitivity was 0.11 ng/mL; the intra-assay CV was 3.2%. The RNA extraction was performed using Trizol according to the manufacturer's instructions, as reported by Fernández-Foren et al. (2023). The concentration and purity of the RNA were determined using a spectrophotometer (NanoDrop ND 1000; Thermo Scientific, Wilmington, DE). Total RNA was treated with DNase using a DNA-free kit (Ambion, Austin, TX). For each sample, cDNA was synthesized by reverse transcription using a SuperScript III transcriptase (Invitrogen) with random primers and 1 µg of total RNA as a template. Real-time PCR (**qPCR**) was performed using a SYBR Green master mix (Thermo Fisher Scientific) and samples were analyzed in a Rotor-Gene 6000 kit (Corbett Life Sciences, Sydney, Australia). The efficiency of the assay was calculated according to Rutledge and Côté (2003). Sequences, the expected product lengths, and efficiency of primers to amplify cDNA of the target genes *ISG15*, progesterone receptor (*PGR*), and the endogenous control β-actin (*ACTB*) were as follows: *ISG15* (NM_001009735.1) forward: GGTATCCGAGCTGAAGCAGTT, reverse: ACCTCCCTGCTGTCAAGGT, 87-bp amplicon, efficiency: 2.04; *PGR* (NM_001205356.1) forward: GACAGCACTTTCTAGGCGACAT, reverse: TGTGCTGGAAGAAACGATTGC, 79-bp, efficiency 2.10; *ACTB* (BT030480) forward: CGTGGCTACAGCTTCACC, reverse: GAAATCGTCCGTGACATCAA, 53-bp, efficiency 1.94. Gene expression was measured by relative quantification to the calibrator (pool of mRNA from each sample enrolled in the study, analyzed in duplicate) and normalized to the endogenous control gene (*ACTB*) using the Pfaffl method, considering the respective amplification efficiencies (Pfaffl, 2001). Sample size calculations were performed using Proc Power (SAS Studio, SAS Institute Inc., Cary, NC). Serum P4 concentrations and gene expression were analyzed by a Glimmix procedure (Proc Glimmix; SAS Studio) for repeated measures having as a basic unit the heifer nested into group, with an autoregressive order 1 correlation structure. Fixed effects included the group, day, and the interaction between group and day. Gene expression data did not follow normal distribution in the Shapiro-Wilk test ($\alpha < 0.05$) and were transformed to natural logarithms. The transformed data were used to calculate *P*-values, whereas the corresponding untransformed LSM and SE are reported for clarity. Significance was considered with $\alpha \leq 0.05$, and tendency between 0.05 and 0.10. According to the differences in the mRNA relative expression between groups, a receiver operating characteristic (**ROC**) curve for the *ISG15* expression on each day and *PGR* on d 18 was generated

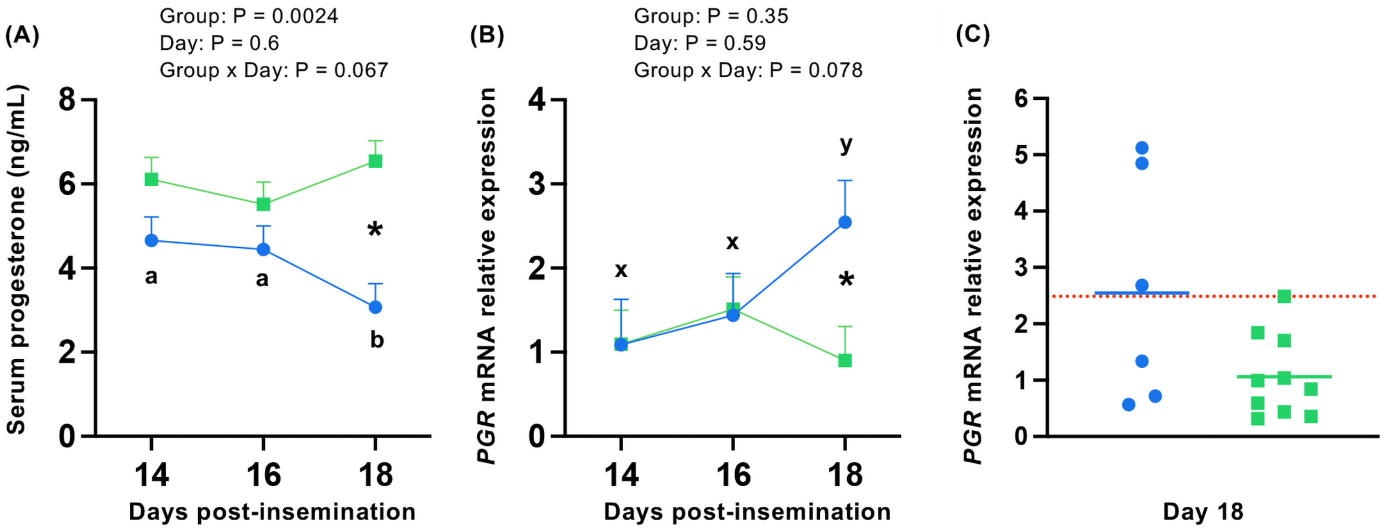

Figure 1. Serum progesterone concentrations (A) and relative mRNA expression of *PGR* (B) on d 14, 16, and 18 postinsemination, and scatter plot with individual distribution of *PGR* relative mRNA expression on d 18 (C) in control (blue) and pregnant (green) heifers. Dots show the individual values. Means are indicated by the continuous midlines. Red dotted lines indicate the threshold for pregnancy diagnosis. *Indicates significant difference between groups on this day. Differences within group among days: a versus b ($P < 0.05$), x versus y ($P < 0.1$). Error bars are SEM.

by JMP 17 software (SAS Institute Inc.). Additionally, numbers of heifers correctly identified as pregnant (true positive [**TP**]), incorrectly identified as pregnant (false positive [**FP**]), correctly rejected as pregnant (true negative [**TN**]), and incorrectly rejected as pregnant (false negative [**FN**]) were calculated in the same package to test performance parameters. Sensitivities, specificities, positive predictive value (**PPV**), negative predictive value (**NPV**), and accuracy were also determined for each day as reported by Pugliesi et al. (2014).

Serum P4 concentrations were greater in pregnant than in control heifers (6.1 ± 0.32 vs. 4.1 ± 0.36 ng/mL, $P = 0.0024$). Even though there was no effect of day, the interaction between group and day tended to be significant ($P = 0.067$): control heifers presented decreased P4 concentrations on d 18 compared with previous days ($P < 0.05$) and pregnant heifers ($P = 0.0007$, Figure 1A). The cervical relative expression of *PGR* mRNA had no significant effect of group or day, but the interaction between group and day tended to be significant ($P = 0.078$), as pregnant heifers maintained their *PGR* cervical expression, whereas control heifers tended to increase *PGR* mRNA expression on d 18 ($P = 0.089$). On d 18 control heifers had greater *PGR* cervical expression than pregnant heifers ($P = 0.031$, Figure 1B). The relative expression of *ISG15* mRNA was greater in pregnant than control heifers (1.89 ± 0.49 vs. 0.13 ± 0.04, respectively, $P < 0.0001$), being different in each day. Pregnant heifers presented 12.0-, 21.5-, and 12.1-fold greater *ISG15* expression on d 14, 16, and 18, respectively, relative to control heifers. The interaction between group and day tended to be significant ($P = 0.059$), as pregnant heifers presented greater *ISG15* mRNA expression on d 16 than on d 14 ($P = 0.0075$), whereas the expression on d 18 was not different from the other days (Figure 2A). No differences among days were detected in control heifers. The ROC curve analysis (Table 1) indicated that *ISG15* relative expression was a significant predictor of pregnancy on d 14 (area under the curve [**AUC**] = 0.86, $P = 0.0027$), d 16 (AUC = 1.0, $P < 0.0001$), and d 18 (AUC = 0.93, $P = 0.0009$), as well as *PGR* relative expression on d 18 (AUC = 0.73, $P = 0.0417$).

The characterization of the physiological scenario through P4 concentrations and *PGR* expression followed the expected results widely described by the literature. Control heifers had decreased P4 concentrations on d 18 when compared with previous days, and on this day it was lower than pregnant heifers, showing the initiation of luteolysis (Lukaszewska and Hansel, 1980). Although we did not find other reports of mRNA *PGR* expression in cervical cells during early pregnancy, in control heifers it tended to increase at the end of the estrous cycle, consistent with the cyclic changes and the cease of the known P4 downregulation on its own receptors as described for the uterus (Meyer et al., 1988; Kimmins and Maclaren, 2001; Meikle et al., 2001). Nevertheless, there was a marked individual variation in cervical *PGR* expression in control heifers on d 18 (Figure 1C), leading to low specificity and lower accuracy of this indicator as a pregnancy diagnostic tool on d 18 (Table 1). The variation in *PGR* expression in control heifers on d 18 could be associated with individual differences in P4 concentrations and timing of luteolysis (Ginther et al., 1989). To our knowledge, this is the first report showing greater *ISG15* mRNA expression in cervical samples as early as d 14 of pregnancy, in comparison to control sham-inseminated heifers. Moreover, as far as we know, this is the first report of the profile of cervical *ISG15* expression in pregnant heifers across days (repeated measures) around maternal recognition of pregnancy. Most of the studies using the traditional methodology of measurement of *ISG15* expression in blood immune cells reported differences from d 18 to 20 of pregnancy (Gifford et al., 2007; Green et al., 2010; Pugliesi et al., 2014; Melo et al., 2020a), with accuracy of pregnancy diagnosis ranging from 70% to 80% in either lactating dairy cows (Han et al., 2006; Yoshino et al., 2018) or beef cattle (Pugliesi et al., 2014; Melo et al., 2020a). It is suggested that tissues with closer proximity to the embryo may have increased expression of ISG

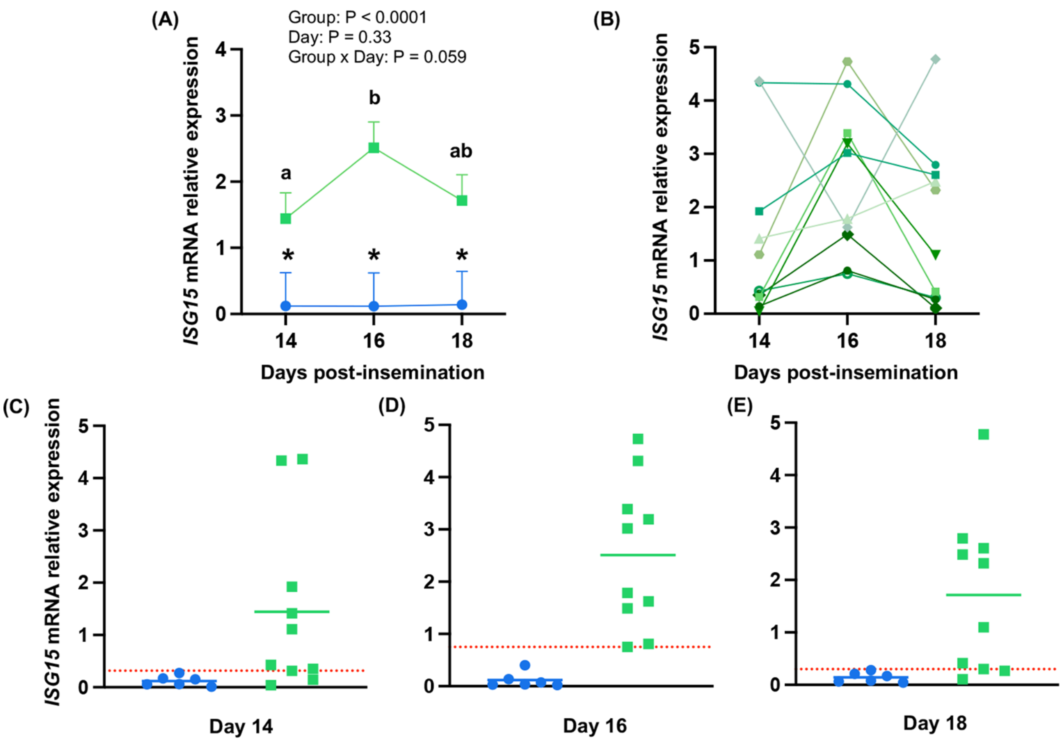

Figure 2. Relative mRNA expression of *ISG15* in control (blue) and pregnant (green) heifers (A) and individual *ISG15* relative mRNA expression of each pregnant heifer (B) on d 14, 16, and 18 postinsemination, and scatter plots with individual distribution of *ISG15* relative mRNA expression in control (blue) and pregnant (green) heifers on d 14 (C), 16 (D), and 18 (E) postinsemination. Dots show the individual values. Means are indicated by the continuous midlines. Red dotted lines indicate the threshold for pregnancy diagnosis. *Indicates significant difference between groups on this day. Differences within group among days: a versus b ($P < 0.05$). Error bars are SEM.

earlier in pregnancy, in concordance with Sponchiado et al. (2017). Other studies found pregnancy-associated greater expression of *ISG15* mRNA in cervix of lactating Holstein cows from d 17 to 18 (Kunii et al., 2018; Domingues et al., 2024) or Holstein heifers and lactating cows on d 20 postinsemination (Ferraz et al., 2021). These studies differed in that pregnant cows were compared with

Table 1. Number of animals, true positive (TP), true negative (TN), false positive (FP), and false negative (FN) diagnoses, and sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, and area under the curve (AUC) for determining pregnancy status on d 14, 16, and 18 post-AI by *ISG15* and on d 18 by *PGR* mRNA relative expression

	ISG15			*PGR*
Item	d 14	d 16	d 18	d 18
No. of animals	16	16	16	16
TP	8	10	8	10
TN	6	6	6	3
FP	0	0	0	3
FN	2	0	2	0
Sensitivity[1] (%)	80.0	100	80.0	100
Specificity[2] (%)	100	100	100	50.0
PPV[3] (%)	100	100	100	76.9
NPV[4] (%)	75.0	100	75.0	100
Accuracy[5] (%)	87.5	100	87.5	81.3
AUC (%)	86.0	100	93.3	73.3
P-value	0.0027	<0.0001	0.0009	0.0417

[1] Sensitivity (probability that a test result will be positive when the cow is pregnant) = TP/(TP + FN).

[2] Specificity (probability that a test result will be negative when the cow is not pregnant) = TN/(FP + TN).

[3] PPV (probability that the cow is pregnant when the test is positive) = TP/(TP + FP).

[4] NPV (probability that the cow is not pregnant when the test is negative) = TN/(FN + TN).

[5] Accuracy = (TP + TN)/n (Pugliesi et al., 2014).

cows also inseminated with regular semen but were not pregnant at ultrasound around d 30. The fold-change found in the present study is lower than other reported previously, between 50- and 80-fold (Kunii et al., 2018; Domingues et al., 2024), but greater than the reported by Ferraz et al. (2021) of 3.4-fold. Differences could be due to the type and day of tissue sampling as well as PCR analysis methodology. The greater fold-change between pregnant versus control heifers in the present study was on d 16 of pregnancy, whereas on d 14 and 18 the lower fold-change was also accompanied by a greater individual variability of *ISG15* expression in the pregnant group (Figure 2C–E, Table 1). On d 16 there was no FP or FN (100% of accuracy), whereas on d 14 and 18 postinsemination the accuracy of the diagnosis was acceptable (87.5% in both days). The AUC reached in the present study were greater than those reported by Ferraz et al. (2021) for the same tissue, which could be explained by the day of sampling and the use of control sham-inseminated animals versus nonpregnant animals that could have undergone early embryo losses around or after the sampling time and before the ultrasonography diagnosis. The good specificity and PPV reached with the present data on all days indicate that this biomarker could be a trustable predictor of the positive pregnancy status; however, FN could exist in animals presenting lower expression, which could represent a risk for iatrogenic pregnancy loss if used as a tool for resynchronization protocols. However, further studies with a larger sample size and the inclusion of nonpregnant animals after AI are necessary to determine the accuracy of this methodology. Also, comparisons between heifers and cows are needed because differences between parities in ISG expression in blood cells have been reported (Green et al., 2010; Melo et al., 2020a). The increased *ISG15* expression particularly on d 16 coincides with the moment of maternal recognition of pregnancy in cattle (Hansen et al., 2017), which seems to be a critical stage in the communication of embryo–uterine–ovarian axis (Forde and Lonergan, 2017). In this sense, it was demonstrated in cattle that transfer of embryos to recipients up to d 16 relative to estrus led to a normal pregnancy, while none of the d 17 recipients were pregnant by d 42 (Betteridge et al., 1980). This is in concordance with the peak found on d 16 of pregnancy in studies of repeated measurements of mRNA expression of IFNT in bovine conceptuses and plasma of pregnant cows (Farin et al., 1990; Sheikh et al., 2018). Other works have found increased *ISG15* expression in the endometrium from d 15 to 17 in pregnant compared with nonpregnant cows (Austin et al., 2004; Moraes et al., 2020; Adhikari et al., 2022). However, an investigation in the dynamic temporal pattern of the endometrial transcriptome of pregnant heifers found differences on d 16 but not on d 13 or before, when compared with cyclic heifers (Forde et al., 2011), in disagreement with Sponchiado et al. (2017), which could be attributed to the localization of the samples, the transcriptomic technique, or both. Nonetheless, the present results sustained the hypothesis that IFNT induces the expression of ISG in surrounding reproductive tissues as early as d 14 of pregnancy, and further research could help to elucidate if it can vary depending on the type of cells collected.

In conclusion, cervical cells express greater *ISG15* mRNA in pregnant versus control heifers as early as d 14 postinsemination, with the best accuracy on d 16. Our findings indicated that the technique proposed might serve as a reliable pregnancy diagnostic tool for Holstein heifers, although further validation with a larger sample size is needed, including animals suffering embryo losses and different parities.

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Notes

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Nonstandard abbreviations used: AUC = area under the curve; CASA = computer-assisted semen analyzer; FN = false negative; FP = false positive; IFNT = interferon tau; ISG = interferon-stimulated genes; NPV = negative predictive value; P4 = progesterone; PGR = progesterone receptor; PPV = positive predictive value; qPCR = real-time PCR; ROC = receiver operating characteristic; TN = true negative; TP = true positive.

Otros artículos asociados a esta tesis

Performance of autumn- and spring-calving Holstein dairy cows confined indoors or managed with pasture and supplementation under various housing conditions. Menegazzi G, **Mendina GR**, Grille L, Méndez MN, Pons V, Pedemonte A, Adrien ML, Meikle A, Gerrits WJJ, Dijkstra J, Chilibroste P. (2025) Journal of Dairy Science. Apr 28:S0022-0302(25)00275-9. doi: 10.3168/jds.2024-26084.

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Performance of autumn- and spring-calving Holstein dairy cows confined indoors or managed with pasture and supplementation under various housing conditions

Gabriel Menegazzi,[1,2]* Graciana R. Mendina,[3] Lucía Grille,[3] Maria Noel Méndez,[3] Victoria Pons,[3] Ana Pedemonte,[4,5] Maria de Lourdes Adrien,[3] Ana Meikle,[6] Walter J. J. Gerrits,[2] Jan Dijkstra,[2] and Pablo Chilibroste[1]
[1]Departamento de Producción Animal y Pasturas, Facultad de Agronomía, Universidad de la República, Paysandú, 60000, Uruguay
[2]Animal Nutrition Group, Department of Animal Sciences, Wageningen University and Research, Wageningen, 6700 AH, the Netherlands
[3]Departamento de Ciencias Veterinarias y Agrarias, Facultad de Veterinaria, CENUR Litoral Norte,
Universidad de la República, Paysandú, 60000, Uruguay
[4]Departamento de Ciencias Sociales, Facultad de Agronomía, Universidad de la República, Montevideo, 12900, Uruguay
[5]Instituto Nacional de la Leche, Montevideo, 11700, Uruguay
[6]Laboratorio de Endocrinología y Metabolismo Animal, Facultad de Veterinaria, Universidad de la República, Montevideo, 13000, Uruguay

ABSTRACT

Dairy cows' productive performance is influenced by various factors, including nutrition, housing comfort, and weather conditions during grazing. In classic confinement systems, animal comfort is typically ensured through ventilation and sprinkling features. However, in mixed feeding systems, controlling environmental conditions at the supplementation site is less common. The present 2-yr study evaluated 2 lactations and 2 calving seasons (autumn and spring) with 3 treatments that combined 2 housing systems (compost barn [CB] or outdoor soil-bedded pens with shade structures [OD]) and 2 feeding strategies with varying proportions of grazing (TMR with no grazing and one-session grazing supplemented with mixed ration [GRZ]). The treatments resulted in the following combinations: (1) CB-confined cows fed a TMR ad libitum (CB-TMR); (2) CB housing with GRZ (CB-GRZ); and (3) OD housing with GRZ (OD-GRZ). Data collected included milk production and composition, feed intake, BCS, BW, and cyclicity. The confined system resulted in higher milk and milk solids production per cow than the mixed feeding systems, with differences being more pronounced in the spring- than in the autumn-calved cows. However, contrary to our hypotheses, the differing environmental exposure in the 2 mixed feeding systems did not affect overall performance, although under certain exposure conditions, the CB outperformed and better protected against heat stress than the OD during several weeks of the trial. In OD-GRZ, milk solids production per cow was greater in autumn- than in spring-calving season, whereas solids production in CB-TMR and CB-GRZ did not differ between autumn- and spring-calving cows. Total DMI and feed efficiency, as well as the amount of concentrate per unit of milk, were higher for the confined system than the mixed feeding systems, with no effect of calving season or environmental exposure level between the 2 mixed feeding systems. Confined cows maintained higher body reserves throughout the trial, with no effects of environmental exposure in the mixed feeding systems. The OD-GRZ had the highest operating profit and return on assets, followed by CB-GRZ and CB-TMR. In conclusion, in the mixed feeding system, performance was not affected by housing conditions at the supplementation site, whereas profitability was higher for OD-GRZ. Regardless of calving season, confined cows produced more milk than mixed feeding cows, with differences being more pronounced in spring-calving compared with autumn-calving cows.

Key words: housing infrastructure, mixed feeding systems, grazing

INTRODUCTION

Pasture-based dairy farming systems are gaining significant attention around the world due to their ability to use land unsuitable for crop production (van Vuuren and Chilibroste, 2013) and convert human nonedible biomass into high-quality protein (Gaughan et al., 2019), thereby contributing to feeding the growing global population (FAO, 2009). Additionally, these systems are well regarded by consumers (Moscovici Joubran et al., 2021), may improve animal welfare (Arnott et al., 2017), and may provide environmental benefits, including carbon

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*Corresponding author: gmenegazzi@fagro.edu.uy

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

sequestration in soils and mitigation of GHG emissions (O'Brien et al., 2015; Aguirre-Villegas et al., 2017; Zubieta et al., 2021) when compared with fully indoor housing systems. Reducing the environmental effect of on-farm dairy production while maximizing resource use efficiency is crucial for sustainable milk production systems (FAO, 2018).

Milk production efficiency in mixed feeding systems is affected by nutrition, housing comfort, and weather conditions. In conventional confinement systems, animal comfort is typically improved through several measures, including ventilation and sprinkling features (Leso et al., 2020). However, in mixed feeding systems, controlling environmental conditions at the supplementation site is less common. In Uruguay, roughage or mixed ration (**MR**) supplementation is predominantly fed in feeders distributed across the field (40%–45%), with a smaller proportion offered in concrete feed pads (22%–26%; Fariña and Chilibroste, 2019). The climate in Uruguay is temperate hot-summer, presenting hot and humid summers and cool to mild winters (Beck et al., 2018). This climate shapes the typical characteristics of Uruguay's grazing dairy systems, which are predominantly pasture-based all year-round, with supplementation, and rely on long-term pastures integrated with annual forage crops in a rotational sequence (Fariña and Chilibroste, 2019). On an annual basis, the diet of dairy herds in Uruguay consists of 56% grazed pasture and 44% roughages plus concentrates, yielding an average of 5,128 L/cow at a stocking rate of 0.99 cows/ha, with calvings concentrated in autumn and spring (INALE, 2019).

Over the past 3 decades, a clear intensification process has taken place in Uruguay, with the number of dairy farmers decreasing by ~50%, the total dairy area shrinking by 29%, the number of cows increasing by 15%, and total milk production by 125% (Chilibroste et al., 2024). More recently, increases in both stocking rates and individual milk production have been accompanied by greater concentrate supplementation (Fariña and Chilibroste, 2019) and increased home-grown forage consumption (Chilibroste et al., 2024). Globally, increased stocking rates have been used to enhance grazed forage utilization per hectare (Doole and Romera, 2013; Romera and Doole, 2015) and are often associated with higher feed supplementation (Patton et al., 2016; Evers et al., 2021). This approach has been shown to improve productivity per hectare and profitability in pasture-based systems (Macdonald et al., 2008; Coffey et al., 2018; Hanrahan et al., 2018; Ortega et al., 2024). However, the intensification of pasture-based dairy systems is also linked to nutrient surpluses (Stirling et al., 2024b; van Loon et al., 2024).

In temperate hot-summer climate regions, higher stocking rates result in cows spending longer periods in adjacent areas, reducing their grazing time on pasture, and limiting grazing opportunities due to grass shortages and extreme weather events, such as heatwaves or the risk of pasture damage after heavy rainfall (Vibart et al., 2012; Ortega et al., 2024). During late spring and summer, it is common practice to restrict cows to grazing only at night due to high daytime temperature humidity index (**THI**) levels, which negatively affect behavior and performance (Polsky and von Keyserlingk, 2017; Román et al., 2017; Morales-Piñeyrúa et al., 2022). When pasture access is restricted, cows need to be supplemented elsewhere in conditions that ensure their welfare. However, confining animals in small areas without proper manure management infrastructure can lead to environmental concerns. The accumulation of urine and feces in confined spaces increases nutrient imbalances, leading to associated risks of N losses (Stirling et al., 2024a) and P contamination in water (Smith et al., 2013; Barreto et al., 2017).

Experimental systems, database analysis, and whole-farm models have critically assessed the intensification process, providing a framework for dairy farmers in the region (Fariña and Chilibroste, 2019; Lazzarini et al., 2019; Stirling et al., 2021a,b; Baudracco et al., 2022; Chilibroste et al., 2024; Ortega et al., 2024; Pedemonte et al., 2024). There is broad agreement that increasing milk production in the region is feasible through higher stocking rates, increased supplementation, and improved utilization of home-grown forage. However, the existing infrastructure on dairy farms is recognized as a limiting factor for both expanding milk production and ensuring cow welfare (Fariña and Chilibroste, 2019; Lazzarini et al., 2019; Chilibroste et al., 2024).

The type of infrastructure adopted to address the aforementioned issues is crucial for dairy farmers, as it affects the entire milk production system (Bewley et al., 2017), even if the infrastructure is intended for part-time use. Compost-bedded packed-barn systems (hereafter referred to as compost barns), with or without access to grazing, have emerged as an alternative to the widely used outdoor soil-bedded systems (i.e., open areas with or without natural or artificial shade where feeders are allocated, which are highly exposed to weather conditions; Leso et al., 2020; Méndez et al., 2020). Compost barns have gained global popularity as a housing system with the potential to improve animal welfare, providing adequate udder health and simplifying manure management (Bewley et al., 2017; Leso et al., 2020). However, limited research is available on the long-term effects of compost barns, spanning one or multiple lactations, on cow production and economic viability, particularly in mixed feeding systems in the temperate hot-summer climate zone of South America.

As part of a broader effort to evaluate the effects of housing conditions and of feeding strategies in pasture-based dairy systems, this study examined the overall effect of housing conditions during nongrazing periods (access to a compost barn or to an outdoor soil-bedded pen) and of feeding strategies (grazing plus MR or full TMR without grazing) on the productive and economic performance of autumn- and spring-calving season dairy cows throughout 2 full lactation periods. Other studies conducted over specific periods within the same general experiment reported here have been published, focusing on welfare (Pons et al., 2023), udder hygiene and mastitis indicators (Mendina et al., 2023), milk fatty acid profile (Grille et al., 2023), metabolic adaptation to lactation (Mendina et al., 2024), behavior, intake, digestion, and milk yield in early lactation (Méndez et al., 2024), and the effect of heat stress on milk production (Méndez et al., 2023). We hypothesized that mixed feeding cows receiving supplementation in a compost barn would produce more milk per lactation than mixed feeding cows receiving supplementation in an outdoor soil-bedded pen, depending on weather conditions. Both mixed feeding systems were compared with a confined system used as a positive control. Finally, we hypothesized that spring-calving cows will produce less milk than autumn-calving cows in the mixed feeding system due to expected more adverse conditions in the first part of lactation.

MATERIALS AND METHODS

A multiyear farmlet-scale experiment was carried out at Estación Experimental "Dr. Mario A. Cassinoni" (EEMAC) of the Facultad de Agronomía – Universidad de la República (Paysandú, Uruguay, 32°S, 58°W) from March 2019 to February 2021. The experimental protocol was approved by the Ethics Committee of the Universidad de la República (protocol number Exp. 020300–000602–18).

Experimental Design, Treatments, and Cows

During the 2-yr study, 2 calving seasons (autumn and spring) were evaluated each year. Sixteen Holstein dairy cows (4 pens) per treatment per calving season per year were used, totaling 192 cows (48 pens). All cows received equal management during both the far-off and close-up periods, fed an anionic precalving diet for ~3 wk before their expected calving dates. Autumn-calving cows averaged a calving date of March 16 ± 9 d in 2019 and March 31 ± 20 d in 2020. Spring-calving cows averaged a calving date of August 10 ± 12 d in 2019 and of July 24 ± 11 d in 2020. Each year, cows were selected from the general experimental herd of the research station and blocked by number of lactations, expected calving date, precalving BW, and precalving BCS. They were randomly assigned to treatments and grouped in pens of 4 animals each after calving. After completing their lactation, cows returned to the general herd and were eligible for reselection if they again met the inclusion criteria, ensuring the formation of groups as homogeneous as possible.

Table 1. The actual days of the experiment and grazing in each year and calving season (CS)

CS	Initial date	Final date	Total days	Days no grazing (confined)[1]
Yr 1				
Autumn	Mar. 21, 2019	Jan. 22, 2020	308	42
Spring	Aug. 8, 2019	May 27, 2020	294	56
Yr 2				
Autumn	Apr. 1, 2020	Dec. 4, 2020	248	47
Spring	Jul. 24, 2020	Feb. 11, 2021	203	8

[1] For the mixed feeding treatments.

An incomplete factorial arrangement was carried out involving 3 treatments. The treatments combined 2 housing systems (compost barn [**CB**] or outdoor soil-bedded pens with shade structures [**OD**]) and 2 feeding strategies with varying proportions of grazing (TMR with no grazing and one-session grazing supplemented with MR [**GRZ**]). The treatments resulted in the following combinations: (1) CB-confined cows fed a TMR ad libitum (**CB-TMR**); (2) CB housing with GRZ (**CB-GRZ**); and (3) OD housing with GRZ (**OD-GRZ**). The treatments began immediately after calving and lasted until the end of the corresponding lactation (Table 1). The CB-TMR and CB-GRZ cows (during out-of-pasture timeslots) were housed in groups of 4/pen, with 13.5 m^2/cow. The barn setup included a concrete area of 6.7 m^2/cow, followed by a feed bunk with a length of 0.75 m/cow. The compost pack was aerated to a depth of 0.35 m 2 times daily using a chisel plow. Every 15 to 20 d, a new layer of sawdust, wood shavings, and rice husks, measuring 9 cm for CB-TMR and 6 cm for CB-GRZ, was added to the bedded pack areas. This addition was based on weekly monitoring of compost temperature and humidity. The concrete area was cleaned 3 times a week by trawling. Effluent from cleaning feed alleys and milking areas was collected but not recirculated to the field during the trial. To cool the cows, the barn was equipped with fans and sprinklers. Automatic sprinklers were activated for 3 min with 10-min nonspray intervals when ambient temperatures exceeded 25°C.

The OD-GRZ cows were kept in groups of 4/pen in OD. Each pen consisted of a total area of 120 m^2/cow, with a central area divided into 2 subpens with an area of 48 m^2/cow. These subpens were alternately occupied based on soil moisture and surface condition. Including the feeding and shade areas, the effective usable area was 72 m^2/cow. Artificial shade, with a nylon roof positioned at a height of 4.5 m with a slope of 15%, offering

4.8 m^2/cow, was provided. The remaining area was left uncovered and therefore exposed to the weather. The effluent from these pens was not collected. The feed bunk, with a length of 1.1 m/cow, was located on the opposite side of the shaded area (Figure 1). For simplicity, CB-GRZ and OD-GRZ may be collectively referred to as mixed feeding cows or mixed feeding systems, whereas CB-TMR may be referred to as confined cows or confined system.

Feeding System and Grazing Management

An identical mixture of silage, hay, and concentrate (the sole feed in the confined treatment and the nongrazing component of the diet in the mixed feeding treatments) was provided, with variations in ingredients occurring over time based on the availability of roughage, market grains, and byproducts. The main components of the ration and their chemical composition are presented in Table 2. All cows had ad libitum access to fresh water through automatic drinkers.

The CB-TMR cows were fed ad libitum 2 times daily. In the mixed feeding treatments, the MR was used to complement the variability in pasture growth rate to better meet the cows' requirements. Pasture allowance was adjusted weekly based on the pasture growth rate in the grazing platform area. The aim was to maintain an average mass of 1,800 kg DM/ha in the grazing platform area, thereby maximizing pasture consumption. In the mixed feeding treatments, a stocking rate of 2.5 lactating cows (~1,600 kg BW) per hectare of grazing platform was used. After the experimental cows completed their lactations, nonexperimental cows were introduced into the system until the next calving season to maintain a consistent stocking rate throughout the year. The pastures used comprised tall fescue (*Lolium arundinaceum*), lucerne (*Medicago sativa*) intermixed with orchard grass (*Dactylis glomerata*), oat (*Avena sativa*), annual ryegrass (*Lolium multiflorum*), and soybean (*Glycine max*). Rotational grazing was employed, with new strips allocated weekly. Multiday strip grazing has been implemented at the research station for several years and adopted by some local farmers as a labor-saving strategy, with no observed negative effects on milk production under the conditions in which it was applied (Menegazzi et al., 2024). In periods where grazing was restricted due to limited pasture growth or severe rainfall events, cows in the mixed feeding treatments were fed full TMR. Grazing and nongrazing days were recorded. Except during the aforementioned situations, from March to October, the cows had 7 h daily pasture access between 0700 and 1400 h and were fed MR after evening milking. From November to February, the cows had 8 h daily pasture access between 1800 and 0200 h and were fed MR after morning milking. The pasture mass and pasture allowance are presented in Table 2.

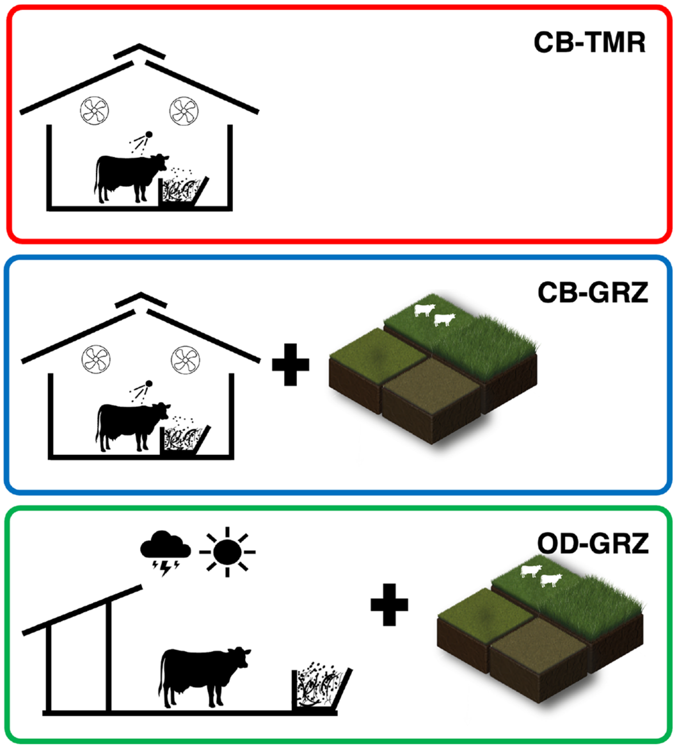

Figure 1. Schematic representation of the treatments: compost barn confinement with TMR (CB-TMR), CB housing with grazing and mixed ration supplementation (CB-GRZ), and outdoor housing with GRZ (OD-GRZ).

Pasture Measurements

Weekly monitoring of the pasture's physiological state (Fulkerson and Donaghy, 2001), sward height, and herbage mass were conducted. The sward height was measured using a sward stick (Barthram, 1985) along zigzag transects. Herbage mass was estimated using a rising plate meter (**RPM**; Ashgrove Co., Palmerston North, New Zealand). Calibration of RPM was conducted every 15 d separately for each treatment by recording the compressed sward height and herbage DM content of 3 contrasting sward heights in triplicate. The compressed sward height was measured with the RPM at each of the 9 sites before cutting the herbage under the RPM to ground level within a 30 × 32 cm frame. The harvested herbage was collected, weighed, and oven-dried at 60°C for 48 h to determine DM content. Individual frame DM herbage mass measurements were regressed against the corresponding RPM compressed sward height to develop herbage mass

Table 2. Ingredient and nutrient composition of the diets, and grazing conditions for compost barn confinement with TMR (CB-TMR), CB housing with grazing and mixed ration (MR) supplementation (CB-GRZ), and outdoor housing with grazing and MR supplementation (OD-GRZ) during whole lactation with autumn- or spring-calving season

	Calving season and treatment					
	Autumn			Spring		
Item	CB-TMR	CB-GRZ	OD-GRZ	CB-TMR	CB-GRZ	OD-GRZ
Ingredients of the TMR or MR						
Corn silage, % DM		20.3			13.4	
Sorghum silage, % DM		9.8			13.2	
Pasture silage, % DM		11.2			13.2	
Concentrate,[1] % DM		58.7			60.2	
Proportions of the diet						
Pasture, % DM	0	35.2	38.8	0	37.7	36.8
MR or TMR, % DM	100	64.8	61.2	100	62.3	63.2
Diet forage:concentrate	41:59	62:38	64:36	40:60	63:37	62:38
Nutrient composition of the diet[2]						
CP, g/kg of DM	163	168	173	164	168	171
NDF, g/kg of DM	354	395	396	372	405	404
ADF, g/kg of DM	187	287	294	201	298	296
Ash, g/kg of DM	86	95	98	89	96	97
Starch,[3] g/kg of DM	202	131	124	186	116	117
$NEL_{3\times}$,[4] Mcal/kg DM	1.52	1.48	1.48	1.51	1.48	1.48
Forage management						
Pasture mass,[5] kg DM/ha	—	2,581	2,578	—	2,726	2,686
Pasture allowance, kg DM/cow/d	—	21	21	—	25	25

[1] Average composition of concentrate (DM basis): corn grain, 22%; wheat bran, 18%; soybean meal, 13%; cottonseed/canola meal, 13%; soybean hulls, 13%; sunflower meal, 11%; rumen-inert fat, 4.2%; calcium carbonate, 2.1%; vitamin-mineral mix, 1.2%; salt, 0.8%; magnesium sulfate, 0.7%; urea, 0.5%; yeast, 0.3%.

[2] For CB-GRZ and OD-GRZ, the nutrient composition of the diet is calculated based on the observed MR intake and estimated pasture intake.

[3] For CB-GRZ and OD-GRZ, the starch content of the diet was calculated assuming starch in the pasture was equal to zero.

[4] $NEL_{3\times}$ = NEL calculated assuming intake at 3× maintenance, according to NRC (2001).

[5] Estimated at ground level.

prediction equations. A pasture sample was collected at the grazing horizon weekly, oven-dried at 60°C for 48 h, stored, and subsequently combined per month for chemical analysis.

Animal Measurements

Individual cow milk yield was automatically recorded 2 times daily at 0400 and 1700 h during spring/summer, and at 0300 and 1600 h during autumn/winter. Individual milk samples were collected weekly from calving until 90 DIM, every 2 wk from 91 until 180 DIM, and monthly until the end of lactation, during both morning and evening milkings. These samples were analyzed for milk fat, protein, and lactose concentrations using midinfrared spectroscopy (MilkoScan FT2, Foss, Drachten, the Netherlands).

The intake of TMR/MR was determined by pen 1 time weekly as the difference between offered and refused feed. On the same day, the TMR/MR was sampled, weighed, and oven-dried at 60°C for 48 h to determine DM content and stored for subsequent chemical analysis. Body weight was recorded monthly. Body condition score was assessed every 2 wk from calving until 120 DIM, and then monthly until the end of lactation, using a 1 to 5 scale (Edmonson et al., 1989). Ovarian ultrasonography was performed on the cows at 21, 40, and 60 DIM to determine cyclicity (presence of corpus luteum; Aloka 500, 7.5 MHz, Tokyo, Japan).

Chemical Analysis

The TMR/MR and pasture samples were analyzed for Ash, CP, NDF, and ADF according to AOAC International (2000). Total nitrogen for CP estimation (nitrogen × 6.25) was determined using the Kjeldahl method described in AOAC (1984), which involves sulfuric acid digestion with subsequent distillation and titration. For NDF (using α-amylase) and ADF, an ANKOM200 Fiber Analyzer (ANKOM Tech. Corp., Fairport, NY) was used. Starch content in the TMR/MR was analyzed according to Hall (2009).

Weather Data and Calculations

Air temperature, relative humidity, and rainfall records were obtained from the meteorological agency of the experimental station every 30 min. The THI was calculated according to NRC (1971) as: (1.8 × AT + 32) − (0.55 − 0.0055 × RH) × (1.8 × AT − 26.8), where AT represents air temperature (°C) and RH represents relative humidity (%).

The Wilmink (1987) exponential function was fitted to daily milk yield data for individual lactations as described below:

$$y_t = a + b \times e^{-kt} + c \times t,$$

where y_t represents the milk yield at day t of lactation, a is the initial milk yield after calving, b is the parameter determining ascending slope before the peak, c is the parameter determining descending slope after the peak, k is a factor related to the moment of peak yield, and e^{-kt} is an exponentially decreasing component. The regression parameters were estimated for each individual lactation using PROC NLMIXED from SAS (SAS Institute Inc., Cary, NC) through 305 DIM for each lactation. Based on the model, the following response variables were obtained: peak milk yield (kg/cow per day), DIM at peak yield, daily milk yield (kg/cow), and 305-d milk yield (kg/cow). Persistency (kg/cow per day after peak) was calculated as the daily milk reduction (kg/d) between peak milk yield and d 305 of lactation.

The TS were obtained by summing the concentrations of fat, protein, and lactose. Energy-corrected milk was calculated according to Sjaunja et al. (1990). Feed nitrogen use efficiency (**NUE**, %) was calculated as the ratio of N excreted in the milk (CP/6.38) to N intake (CP/6.25; Powell et al., 2010).

The income over feed costs was calculated with milk prices based on solids content (INALE, 2021) at \$0.30, \$0.29, and \$0.29/kg of milk for CB-TMR, CB-GRZ, and OD-GRZ, respectively. Feed prices of concentrate, corn silage, sorghum silage, pasture silage, and grazed pasture were \$0.28, \$0.09, \$0.11, \$0.11, and \$0.05/kg of DM, respectively (INALE, 2021). The feed efficiency variables, including ECM per kilogram of DM, gram of concentrate per kilogram of ECM, NUE, and income over feed costs, were calculated with the observed milk production data rather than the predicted milk production.

Pasture intake was estimated using the back-calculation approach, calculated as the difference between NEL consumed and required, following guidelines and equations of NASEM (2021). Briefly, this method involved determining the kilograms of pasture required to meet the remaining energy required to fulfill the cows' NEL requirements, after subtracting the NEL obtained from MR intake. The energy concentration of the feeds offered to the animals was estimated according to NRC (2001), on a weekly basis for the MR and monthly for the grazed pasture. The energy requirements for maintenance, activity (walking and grazing), pregnancy, and milk production were calculated weekly, on a per-pen basis. The cows walked 1.90 km/d horizontally and 0.05 km/d vertically. This distance includes the average round trip from the milking parlor to the pasture.

Economic Analysis

The economic performance of each treatment was simulated considering the necessary investments and costs to achieve the targeted milk production outcomes (experimental results), using the average Uruguayan dairy farm (**AVG-UY**) as a baseline (Table 3 and Appendix Table A1). The AVG-UY was characterized by a national survey conducted by INALE (2014). The simulations were based on prices of the agricultural year 2019/2020 (July 1, 2019 to June 30, 2020). The rationale behind the calculations was to simulate the necessary adjustments on the average farm to match the production levels of the experimental treatments. The targeted milk production for each treatment was the average of both calving seasons, predicted by the Wilmink function. The primary investments considered were the construction of a CB (for CB-GRZ and CB-TMR) and other infrastructure, machinery, and acquisition of (extra) cows. The main costs were associated with feed supply (concentrates). Farm operating profit per hectare was calculated as proposed by Ferris and Malcolm (1999): Farm operating profit = Farm gross income − economic costs (cash expenses plus depreciation) − calculated family labor (opportunity costs)/total farmland. Return on assets was calculated as farm operating profit/total assets (including owned land) × 100. All results are presented in US dollars.

COVID-19 Implications

Due to the COVID-19 pandemic, lockdowns were implemented in Uruguay during 2020 and 2021 while the experiment was underway. At certain moments, the situation was further complicated by a shortage of personnel resulting from strict mandatory isolation rules for those showing COVID-19 symptoms. As a result, ovarian ultrasonography at 40 DIM for the 2020 autumn-calving season cows was skipped. Similarly, the harvesting of crops and pastures for conserved roughage production was compromised. The limited amount of roughage available led to the interruption of the 2020 autumn-calving season

Table 3. Land use, herd characteristics, productive performance, and economic performance in the targeted year used in the economic analysis for compost barn confinement with TMR (CB-TMR), CB housing with grazing and mixed ration supplementation (CB-GRZ), and outdoor housing with GRZ (OD-GRZ)[1]

	Treatment			
Item	CB-TMR	CB-GRZ	OD-GRZ	AVG-UY
Land				
Total area, ha	194	194	194	194
Total area for dairy, ha	160	160	160	160
Total area for adult cows, ha	120	120	120	120
Animal				
Milking cows	281	188	188	104
Adult cows	338	226	226	128
Milking:adult cows ratio	0.83	0.83	0.83	0.81
BW, kg/milking cow	620	606	599	530
Stocking rate, kg BW/ha adult cows	1,749	1,142	1,130	568
Productive performance				
Milk production, kg/yr	3,537,820	1,910,221	1,910,221	730,162
Meat production, kg/yr	59,262	37,560	37,213	23,285
Fat + protein, %	7.04	6.96	6.90	7.20
Stocking rate, adult cows/ha adult cows	2.82	1.89	1.89	1.07
Daily milk production, kg/milking cow	34.6	27.9	27.9	19.24
Milk production, kg/adult cow/yr	10,467	8,452	8,452	5,682
Land productivity, kg milk/ha adult cows/yr	29,509	15,933	15,933	6,090
Labor				
Family workers	2.4	2.4	2.4	2.4
Hired workers	4.4	3.2	3.2	1.8
Total workers	6.8	5.6	5.6	4.2
Family labor, % of total labor	35	43	43	57
Labor productivity, kg milk/worker/yr	520,268	341,111	341,111	173,199
Adult cows/worker	50	41	41	30
Economic performance				
Gross income, US$/ha	5,568	2,976	2,990	1,288
Economic costs, US$/ha	5,278	2,669	2,473	1,039
Operating profit, US$/ha	290	307	517	249
Assets, US$/ha	13,625	10,546	7,361	5,874
Return on assets, %	2.1	2.9	7.0	4.2

[1] The simulations considered the necessary investments and costs to achieve the targeted milk production outcomes from experimental results, using the average Uruguayan dairy farm (AVG-UY; INALE, 2014) as a baseline.

treatments at 250 DIM and the 2020 spring-calving season CB-TMR treatment at 150 DIM, although allowing the mixed feeding treatments to continue until 200 DIM.

Data Editing and Statistical Analysis

All response variables collected or estimated at equally spaced intervals were summarized at the cow or pen level, according to the experimental unit. Milk composition was first summarized monthly and then averaged at the cow level. For statistical analysis, milk production, milk composition, milk solids yield, BCS, and BW variables were included with one value per cow, except for BCS throughout lactation, which was analyzed without further summarization, using 14-d intervals until 150 DIM and 28-d intervals until 305 DIM. The milk production analyzed was that predicted by the Wilmink function.

In the CB-TMR treatment during the autumn-calving season of 2019, 16 cows were excluded from the analyses as a result of a massive outbreak of mastitis caused by *Serratia* spp. at 150 DIM. The infection was chronic and difficult to treat, leading to the interruption of the lactation in several cows to allow for appropriate treatment, following the recommendations of veterinary assistance. The 16 cows of CB-TMR treatment in the spring-calving season in year 2020 were excluded from the analyses because lactation was interrupted at 150 DIM (for details, see the COVID-19 Implications section). Eleven cows were removed due to Wilmink parameter estimates showing a reversed sign or falling outside the expected range, resulting in an abnormal lactation curve shape. Another 18 cows were removed from the dataset due to health problems either at calving or during lactation, following standardized protocols including veterinary advice. The final dataset included 131 individual lactations distributed as follows: autumn-calving season, year 2019: n = 15 for CB-GRZ (4 pens) and 15 for OD-GRZ (4 pens); autumn-calving season, year 2020: n = 13 for CB-TMR (4 pens), 14 for CB-GRZ (4 pens), and 11 for OD-GRZ (4 pens); spring-calving season, year 2019: n = 13 for

CB-TMR (4 pens), 12 for CB-GRZ (4 pens), and 12 for OD-GRZ (4 pens); spring-calving season, year 2020: n = 13 for CB-GRZ (4 pens) and 13 for OD-GRZ (4 pens).

All data were analyzed using the GLIMMIX procedure of SAS. The cow was considered the experimental unit for milk production and composition-, reproduction-, BCS-, and BW-related variables, and the pen was the experimental unit for the DMI- and efficiency-related variables. For individual animal-related variables, analyzing data at either the cow or pen level yielded approximately identical treatment means and no meaningful differences in *P*-values. Unless stated otherwise, all variables were analyzed with a normal distribution and a general model that included treatment, calving season, the interaction between treatment and calving season, and year as fixed effects, with block as random effect. For the BCS and BW variables, the values at calving were added as covariates. For the analysis of BCS throughout the lactation, DIM and its triple interaction with treatment and calving season were added to the fixed effects, and cow was included as a random effect and treated as a repeated measure in the model. The proportion of cows with a corpus luteum was analyzed with a binomial distribution, with cow included as a random effect and treated as a repeated measure to account for observations at 21, 40, and 60 DIM. For both BCS throughout the lactation and proportion of cows with a corpus luteum, the covariance structure variance components provided best fit with the lowest overall Akaike information criterion values. The model assumptions were evaluated using studentized residual plots. Differences between LSM were tested using the Tukey-Kramer test. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Weather Conditions and Actual Days of Grazing

The monthly rainfall during the entire experimental period (Appendix Figure A1), as well as a comparison with the last 15-yr average of rainfall (Appendix Table A2), indicate generally dry conditions during the 2-yr study. The monthly average temperature varied between 10.1°C and 24.8°C, with a minimum of −1.1°C and a maximum of 38.4°C. The monthly average THI during the hottest months (December, January, and February) averaged 71.0 (range 69.6–72.3), with maximum THI reaching 88.4 (Appendix Figure A2). The actual days of the experiment varied between 203 (spring year 2) to 308 (autumn year 1; Table 1). Nongrazing days (between 8 and 56 d/season) primarily occurred due to a shortage of pasture caused by low pasture growth rates related to adverse weather conditions.

Milk and Solids Production

Table 4 presents the effects of treatments throughout the entire lactation period, with either autumn- or spring-calving seasons, on milk production and composition, milk solids production, DMI, feed efficiency, and BCS. Estimated milk yield parameters from the Wilmink exponential function are presented in Appendix Table A3. Individual 305-d milk yield, daily milk yield, and peak milk yield were affected ($P \leq 0.024$) by a treatment by season interaction. Cows in the confined system, compared with cows in the mixed feeding systems, exhibited a greater production in spring- than in autumn-calving season (+31% and +17%, respectively). Similarly, cows in the confined system, compared with cows in the mixed feeding systems, had a more pronounced greater peak milk yield in spring-calving than in autumn-calving season (+28% and +8%, respectively). Spring-calved cows reached peak milk yield earlier ($P < 0.001$), in wk 6 of lactation, whereas autumn-calved cows reached it in wk 10. Additionally, mixed feeding cows reached peak milk yield 17 d earlier than confined cows ($P = 0.004$). Regarding lactation persistency, the milk production after peak decreased by 0.42 kg/wk irrespective of treatment, calving season, or its interaction. Figure 2 depicts the effect of the treatments on the shape of the lactation curve. Fat, protein, and total milk solids concentration did not show significance for any of the tested effects. Lactose concentrations were higher ($P = 0.005$) for CB-TMR than CB-GRZ (4.96% vs. 4.87%). The ECM production was 6.3 kg/d higher for confined cows than mixed feeding cows ($P < 0.001$).

Fat yield at 305 d was 28% higher ($P < 0.001$) for confined than mixed feeding cows. Also, in the OD-GRZ treatment, autumn-calved cows tended ($P = 0.078$) to produce 12% more fat at 305 d than spring-calved cows (treatment by season interaction; $P = 0.094$). In the spring-calving season, the differences in protein, lactose, and total milk solids 305-d yield between confined and mixed feeding cows were approximately double those observed in the autumn season, with protein, lactose, and total milk solids showing differences of 34%, 35%, and 35%, respectively, in the spring-calving season, compared with 16%, 18%, and 18% in the autumn-calving season. Additionally, in the OD-GRZ treatment, autumn-calved cows produced 14% more protein and 12% more total milk solids than spring-calved cows.

DMI and Efficiency

Throughout the entire lactation period, average daily DMI was 2.3 kg/cow higher for confined cows compared with mixed feeding cows ($P = 0.002$). Figure 3 illustrates the DMI of MR and pasture by mixed feeding

Table 4. Milk production and composition, milks solids production, DMI, feed efficiency, and BCS for compost barn confinement with TMR (CB-TMR), CB housing with grazing and mixed ration supplementation (CB-GRZ), and outdoor housing with GRZ (OD-GRZ) during whole lactation with autumn- or spring-calving season

	Calving season (CS) and treatment (TRT)										
	Autumn			Spring				Probability			
Item	CB-TMR	CB-GRZ	OD-GRZ	CB-TMR	CB-GRZ	OD-GRZ	SEM^{1}	TRT	CS	TRT × CS	N
Milk production											
305-d milk yield, kg/cow	10,313^{A}	8,735^{B}	8,949^{B}	10,683^{A}	8,240^{B}	8,065^{B}	267.0	<0.001	0.263	0.024	131
Daily milk yield, kg/cow	33.3^{A}	28.7^{B}	29.4^{B}	35.8^{A}	27.1^{B}	26.4^{B}	0.82	<0.001	0.413	0.002	131
Peak milk yield, kg/cow/d	38.2^{AB}	35.4^{BC}	35.5^{BC}	43.1^{A}	34.0^{BC}	33.5^{C}	1.07	<0.001	0.672	<0.001	131
DIM at peak yield	75.4^{a}	57.6^{b}	59.1^{b}	50.0^{a}	34.6^{b}	33.5^{b}	5.60	0.004	<0.001	0.944	131
Persistency, kg/cow/d after peak	−0.045	−0.065	−0.057	−0.071	−0.059	−0.059	0.0075	0.712	0.372	0.060	131
Milk composition											
Fat, %	3.75	3.62	3.56	3.61	3.60	3.53	0.071	0.180	0.310	0.718	131
Protein, %	3.38	3.33	3.39	3.34	3.36	3.31	0.040	0.919	0.436	0.151	131
Lactose, %	4.98^{a}	4.85^{b}	4.91^{ab}	4.94^{a}	4.88^{b}	4.91^{ab}	0.030	0.005	0.783	0.467	131
Solids total, %	12.11	11.80	11.86	11.87	11.85	11.75	0.101	0.180	0.263	0.378	131
Milk solids yield											
Fat, kg/cow/305 d	385^{a}	317^{b}	318^{b}	393^{a}	296^{b}	284^{b}	9.7	<0.001	0.116	0.094	131
Protein, kg/cow/305 d	345^{A}	290^{BC}	303^{B}	365^{A}	278^{BC}	266^{C}	7.8	<0.001	0.251	0.001	131
Lactose, kg/cow/305 d	511^{A}	425^{B}	440^{B}	538^{A}	403^{B}	396^{B}	12.2	<0.001	0.294	0.015	131
Total milk solids, kg/cow/305 d	1,238^{A}	1,032^{BC}	1,061^{B}	1,294^{A}	976^{BC}	946^{C}	27.6	<0.001	0.193	0.006	131
DMI and efficiency											
DMI, kg DM/cow/d	24.8^{a}	22.8^{b}	22.9^{b}	25.2^{a}	22.6^{b}	22.5^{b}	0.68	0.002	0.851	0.828	48
ECM, kg/cow/d	32.7^{a}	28.7^{b}	29.1^{b}	36.8^{a}	28.6^{b}	27.5^{b}	1.10	<0.001	0.383	0.095	40
Feed efficiency, kg ECM/kg DMI	1.45^{a}	1.25^{b}	1.26^{b}	1.33^{a}	1.26^{b}	1.22^{b}	0.033	<0.001	0.083	0.229	40
Concentrate used/unit milk, g concentrate/kg ECM	417^{a}	292^{b}	285^{b}	430^{a}	277^{b}	288^{b}	11.4	<0.001	0.999	0.465	40
NUE,^{2} %	32.3^{A}	25.1^{C}	25.5^{BC}	27.5^{B}	25.3^{BC}	24.8^{C}	0.48	<0.001	<0.001	<0.001	40
Income over feed costs, US$/cow/d	5.28	5.31	5.57	5.58	5.39	5.34	0.323	0.919	0.849	0.733	40
BCS and BW											
BCS at calving	3.09	3.11	3.10	2.99	3.03	2.90	0.054	0.180	0.021	0.297	131
Minimum BCS during lactation	2.64^{a}	2.53^{b}	2.55^{ab}	2.64^{a}	2.55^{b}	2.59^{ab}	0.031	0.008	0.449	0.905	131
BW at calving	625	642	643	632	619	606	19.0	0.801	0.451	0.264	131
BW at end	692^{a}	634^{b}	653^{b}	662^{a}	623^{b}	620^{b}	10.9	<0.001	0.012	0.508	131

[a,b] Means within a row with different lowercase letters differ ($P < 0.05$) between treatments independent of season.

[A–C] Means within a row with different capital letters differ ($P < 0.05$) between treatments and calving season.

[1] Average SEM is listed.

[2] NUE = feed nitrogen use efficiency.

cows throughout lactation for autumn- and spring-calved cows. On average, mixed feeding cows consumed 13.7 kg DM/cow per day of MR and 8.9 kg DM/cow per day of pasture. The feed efficiency was 11% higher ($P < 0.001$) for confined than mixed feeding cows, and tended to be 4% higher ($P = 0.083$) in the autumn-calving season compared with the spring-calving season. The use of concentrate per kilogram of ECM was 48% higher ($P < 0.001$) for confined cows compared with mixed feeding cows. For NUE, a significant treatment × season interaction ($P < 0.001$) was present. For CB-TMR autumn–calved cows, NUE was higher compared with all other treatments, whereas CB-TMR spring-calved cows had higher NUE than CB-GRZ autumn-calved cows and OD-GRZ spring-calved cows, and tended to be higher than OD-GRZ autumn-calved cows ($P = 0.085$) and CB-GRZ spring-calved cows ($P = 0.058$). The income over feed costs did not show significant differences for any tested effect, averaging $5.41/cow per day.

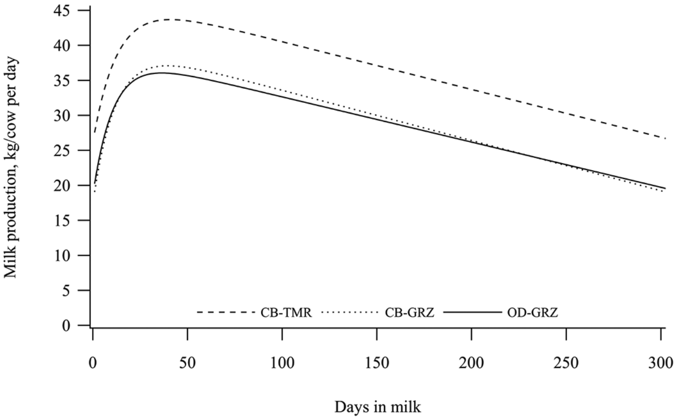

Figure 2. Effects of compost barn confinement with TMR (CB-TMR), CB housing with grazing and mixed ration supplementation (CB-GRZ), and outdoor housing with GRZ (OD-GRZ) on the lactation profile for milk yield (kg/cow per day).

BCS, BW, and Ovarian Cyclicity

There was no calving season by treatment interaction for all BCS and BW variables (Table 4). The average BCS at calving was 3.04 with no treatment effect. However, in the spring-calving season, the BCS at calving was 0.13 units lower than in the autumn-calving season ($P = 0.021$). Minimum BCS during lactation was higher ($P = 0.008$) for CB-TMR than CB-GRZ (2.64 vs. 2.54) and tended to be higher ($P = 0.088$) than OD-GRZ (2.57). The mixed feeding treatments did not differ from each other. Figure 4 depicts the effect of the treatments on BCS throughout the lactation for autumn- and spring-calving season. There was a treatment by calving season by DIM interaction ($P < 0.001$). The confined cows started to recover BCS earlier than the mixed feeding cows, and there was no difference between the mixed feeding treatments. In the autumn-calving season, confined cows had greater BCS than mixed feeding cows at 92, 141, 169, 197, 225, and 253 DIM ($P \leq 0.024$). In the spring-calving season, confined cows had greater BCS than mixed feeding cows at 197 and 281 DIM ($P \leq 0.003$), whereas at 253 DIM, only CB-TMR and OD-GRZ differed from each other ($P < 0.001$). The BW at calving was consistent across treatments, averaging 628 kg (Table 4). The BW at the end of lactation was higher ($P < 0.001$) for confined than mixed feeding cows. Additionally, for autumn-calving season cows, the BW at the end of lactation was ~25 kg higher ($P = 0.012$) than for spring-calving season cows. There was no effect of calving season ($P = 0.197$) or its interaction with treatment ($P = 0.362$) on the proportion of cows with a corpus luteum in the first 60 DIM. However, this proportion was lower ($P < 0.001$) for CB-TMR (31% ± 5.5%) compared with CB-GRZ (56% ± 5.9%) and OD-GRZ (61% ± 6.0%).

Economic Performance

The AVG-UY, used as a baseline for the economic analysis, had approximately half the gross income and economic costs of CB-GRZ and OD-GRZ, and a quarter of those of CB-TMR. Its operating profit was the lowest compared with the experimental treatments, whereas its return on assets was intermediate, higher than that of CB-TMR and CB-GRZ but less than that of OD-GRZ (Table 3). The gross income for confined cows was double that of mixed feeding cows. Economic costs followed a similar pattern, with a difference of $196/ha between the mixed feeding treatments, favoring OD-GRZ. The OD-GRZ exhibited the highest operating profit, being $210/ha higher compared with CB-GRZ, and $227/ha higher compared with CB-TMR. Total assets were almost double in CB-TMR compared with OD-GRZ, and intermediate in CB-GRZ. The return on assets was much higher for OD-GRZ (7.0% of total assets) than for CB-TMR and CB-GRZ (2.1% and 2.9% of total assets, respectively).

DISCUSSION

As part of a broader effort to assess the effect of housing conditions and feeding strategies in pasture-based dairy systems, this study evaluated how different nongrazing housing conditions (CB vs. OD) and feeding strategies (grazing plus MR vs. confined) influence the productive and economic performance of autumn- and spring-calving dairy cows throughout 2 full lactation cycles. This is the first long-term study in the temperate hot-summer

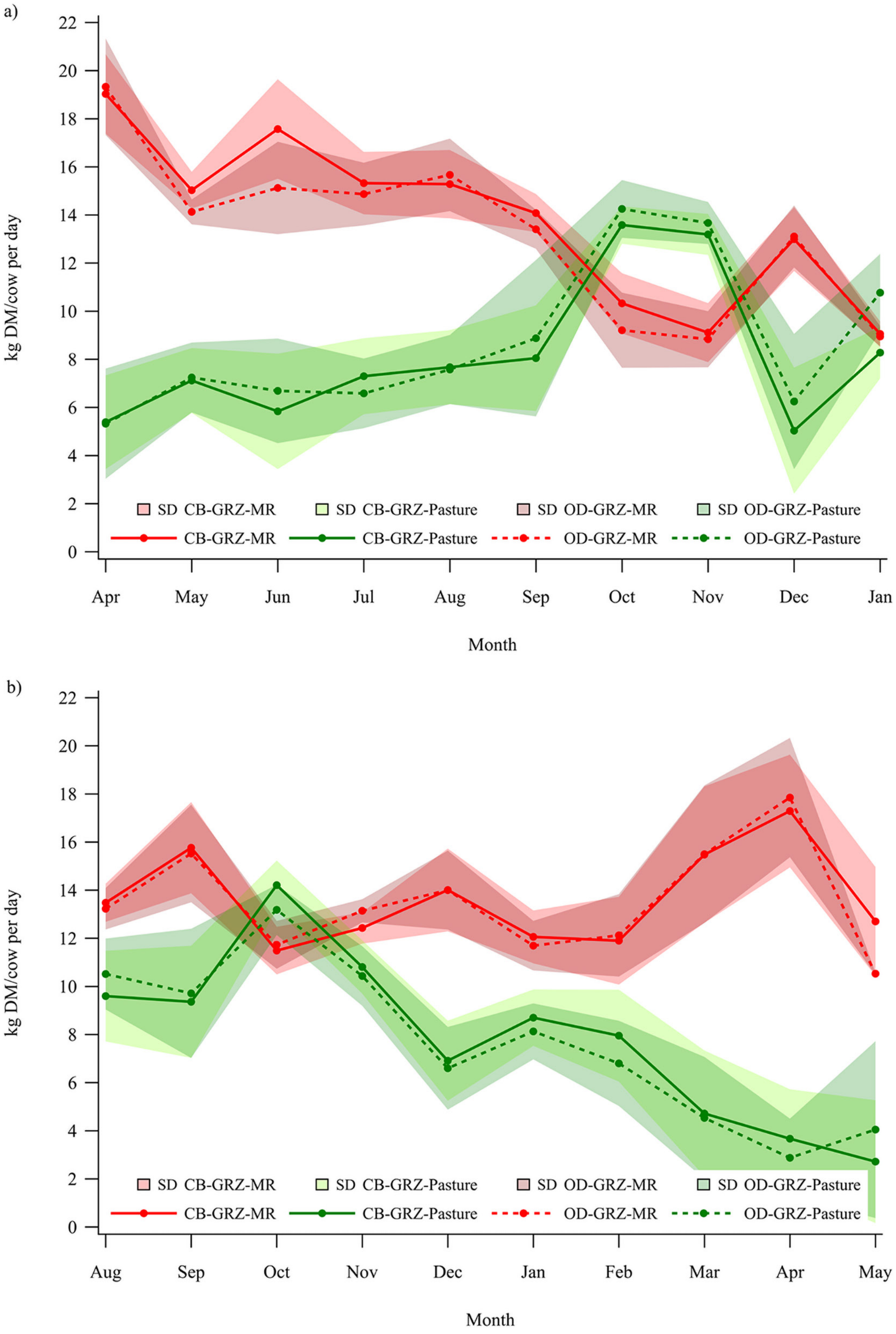

Figure 3. The DMI of mixed ration (MR) and pasture by the compost barn housing with grazing and MR supplementation (CB-GRZ) and outdoor housing with GRZ (OD-GRZ) throughout the lactation for autumn- (a) and spring-calving season cows (b). The average intake of MR and pasture was 13.9 and 8.8 kg DM/cow per day, respectively, for autumn-calving season cows, and 13.4 and 8.9 kg DM/cow per day, respectively, for spring-calving season cows.

climate zone of South America to examine the effects of housing conditions during supplementation, addressing a critical knowledge gap in pasture-based dairy farming and providing valuable insights for decision making. The findings contribute to the ongoing discussion on the intensification of pasture-based dairy systems and the role of infrastructure investments in maintaining sustainable production. Whereas the study primarily focuses on productive and economic performance, it also contributes relevant perspectives on animal welfare and environmental considerations. A key strength of this research is its 2-yr duration, which accounts for interannual weather variations and strengthens the reliability of the results. However, the findings are specific to the conditions observed, and caution is advised when applying these findings to longer periods or different climatic contexts. Additionally, the substantial data removal is an important limitation of the study.

The confined system resulted in higher production of milk and milk solids than mixed feeding systems, with differences being more pronounced in the spring- than in the autumn-calving season. However, in contrast to our hypotheses, environmental exposure differences between the 2 mixed feeding systems did not affect overall performance. Nevertheless, depending on actual exposure conditions, the CB provided better performance and heat stress protection for several weeks of this trial compared with OD (Méndez et al., 2023). Total DMI and feed efficiency as well as concentrate used per unit milk were higher in the confined system than in the mixed feeding systems, with no effect of calving season or environmental exposure level between the 2 mixed feeding systems, except for a tendency to greater feed efficiency in the autumn-calving season. Milk solids production in the OD-GRZ system was greater in autumn- than in spring-calving season, whereas solids production in CB-TMR and CB-GRZ did not differ between autumn- and spring-calving cows. Confined cows maintained higher body reserves throughout the trial, with no effects of environmental exposure in mixed feeding systems.

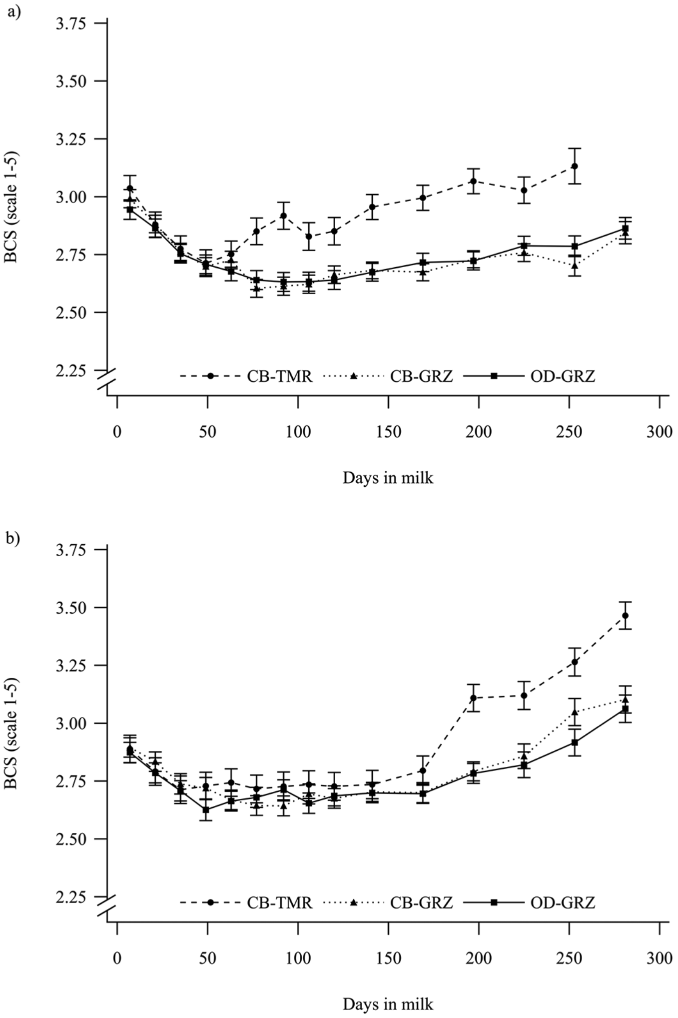

Figure 4. Effects of compost barn confinement with TMR (CB-TMR), CB housing with grazing and mixed ration supplementation (CB-GRZ), and outdoor housing with GRZ (OD-GRZ) on BCS throughout the lactation for autumn- (a) and spring-calving season cows (b). Error bars represent SEM.

Housing Conditions of Mixed Feeding Treatments

Stocking rate is a crucial factor in enhancing productivity per hectare and profitability in pasture-based systems worldwide (Macdonald et al., 2008; Coffey et al., 2018; Hanrahan et al., 2018; Ortega et al., 2024). However, in Uruguay, higher stocking rates lead to more time cows spent off pasture to receive supplements, due to reduced pasture availability per cow (Ortega et al., 2024). A similar pattern was reported by Vibart et al. (2012) for the Eastern United States, where the greater stocking rate increased supplementation time spent in adjacent feeding areas. Contrary to our hypothesis, the different environmental exposure at the site where mixed feeding cows were kept during the out-of-pasture times did not affect overall lactation performance. The annual rainfall during the study period was 31% lower than the last 15-yr average, with most months experiencing below-average rainfall. In a typical year with greater rainfall, cows in the OD-GRZ treatment might have faced muddier conditions in the pens, potentially affecting their well-being and performance (O'Driscoll et al., 2008). Both the temperature and THI data suggest that cows may have faced heat stress at some points, depending on the efficiency of the infrastructure provided to mitigate these effects. Méndez et al. (2023) reported higher milk production for CB-GRZ than OD-GRZ spring–calved cows during heat stress waves in the summer during the first year of study. The full lactation approach used in the present analysis could have masked differences in milk production at specific events. When examining only days with an average daily THI above 72 (79 d), the arithmetic mean daily milk yield favored CB-GRZ to OD-GRZ by ~0.78 kg/cow per day in the autumn-calving cows and 1.67 kg/cow per day in the spring-calving cows (data not shown). This suggests that the OD-GRZ infrastructure was inadequate to mitigate heat stress, as indicated by the greater subcutaneous temperature observed in the OD-GRZ cows compared with the CB-GRZ cows during summer (Pons et al., 2023). The stage of lactation when cows encounter challenging environmental conditions effects the extent of productive performance loss (Polsky and von Keyserlingk, 2017). Autumn-calved cows entered summer with lower production levels than spring-calved cows. Whereas not significant, the mixed feeding systems produced 8% less milk in the spring-calving season than in the autumn-calving season. This difference was numerically more pronounced in the OD-GRZ treatment, reaching 10%. In OD-GRZ, autumn-calving cows produced 14% more protein and 12% more TS, respectively, than spring-calved cows, a difference not observed in CB-GRZ cows.

Fariña and Chilibroste (2019) and Ortega et al. (2024) identified the current infrastructure of dairy farms in Uruguay as a potential limit to increasing milk production in mixed feeding systems. However, our results suggest that high performance is achievable in mixed feeding systems (>8,000 kg/cow in 305 d) with relatively low-cost infrastructure (OD-GRZ), providing cows with basic comfort, such as adequate space, shade, ad libitum water supply, and OD maintenance. Stirling et al. (2021a) reported a similar performance of 8,065 kg of milk/cow in 305 d for North American Holstein Friesian cows with comparable housing conditions to OD-GRZ and approximately the same pasture proportion in the diet (37% of DM). Beyond milk production and using the same cows as in the present study, Mendina et al. (2023) evaluated udder hygiene scores throughout the entire lactation during the first year of the study and observed reduced udder hygiene in OD-GRZ cows compared with CB-GRZ cows. Although this difference was not reflected by differences in individual SCC or intramammary infection, the lower udder hygiene could explain higher psychrotrophic bacteria levels in OD-GRZ milk (data not shown). Psychrotrophs significantly affect the deterioration of milk and milk products, resulting in substantial losses for the dairy production chain and negatively affecting dairy product quality (Saha et al., 2024). During spring/summer, Grille et al. (2023) observed in cows of the present study higher α-linolenic acid (C18:3 n-3) proportions in total milk fatty acids in CB-GRZ cows compared with OD-GRZ cows. This finding is significant, as some PUFA in milk benefit human health (Moscovici Joubran et al., 2021). Additionally, Pons et al. (2023) reported lower subcutaneous temperatures and tendency to lower serum creatine kinase concentrations in CB-GRZ than in OD-GRZ cows, whereas Mendina et al. (2024) reported BHB concentrations in OD-GRZ cows that were compatible with subclinical ketosis. Pons et al. (2023) and Mendina et al. (2024) concluded that OD-GRZ cows experienced poorer welfare than CB-GRZ cows during summer, based on such behavioral and physiological indicators.

Grazing provides considerable animal welfare benefits (Kilgour, 2012; Arnott et al., 2017), but concerns persist about mitigation welfare issues from extreme weather events (West, 2003; Arnott et al., 2017; McDowell et al., 2022). In Uruguay, recent studies highlighted negative effects of heat stress on animal welfare, behavior, and milk production (Román et al., 2017; Morales-Piñeyrúa et al., 2022). Moreover, national evidence suggests that shade alone is insufficient to prevent heat stress and its associated negative effects on behavior, welfare (Pons et al., 2023), and performance (Román et al., 2017; Méndez et al., 2023).

Concentrating animals in a small area without proper infrastructure for manure management, as in the case of OD-GRZ infrastructure, may expose the system to significant nutrient imbalances (van Vuuren and Chilibroste, 2013). Fariña and Chilibroste (2019) and Stirling et al. (2024a) highlighted point-source pollution as a major challenge for the expanding dairy farming systems in Uruguay. Indeed, worldwide, a positive association has been reported between the intensification of pasture-based dairy systems and nutrient surpluses, as observed in Australia (Gourley et al., 2012; Smith et al., 2013) the Netherlands (van Loon et al., 2024), Ireland (Adenuga et al., 2020), and Uruguay (Stirling et al., 2024b). In this context, whereas OD-GRZ infrastructure supports similar milk production levels to CB-GRZ, it presents challenges in managing nutrient surpluses, as collecting manure or effluent is more difficult. In contrast, CB-GRZ infrastructure facilitates the collection of a substantial portion of daily excreta, which can be uniformly redistributed, helping to mitigate nutrient losses from the system (McDowell et al., 2022).

Confined Versus Mixed Feeding Systems

Autumn-calving and spring-calving cows on the CB-TMR treatment produced 15% and 34% more milk in a 305-d period, respectively, than autumn-calving and spring-calving mixed feeding cows. These findings are generally consistent with literature, although the magnitude of the differences varies, and few long-term studies covering full lactations have been conducted. The disparity between confined and mixed feeding systems is typically related to factors such as DMI and nutrient content of the diet. For example, Fajardo et al. (2015) reported a 10% increase in milk production for cows consuming 26.1 kg of TMR compared with those consuming 14.3 kg/cow per day of MR and 7.5 kg/cow per day of pasture during the first 10 wk of lactation (37.0 kg of milk/cow per day vs. 33.9 kg of milk/cow per day). Because this period corresponds to early lactation, differences in tissue mobilization likely attenuated the production gap. Bargo et al. (2002) observed a 19% increase in milk production (6.1 kg/d) during a 21-wk trial with mid-lactation cows for those on a full TMR diet (26.7 kg DMI/cow per day) compared with cows fed a combination of TMR and pasture (25.2 kg DMI/cow per day, including 7.5 kg DM/cow per day of pasture). The increased milk production for CB-TMR cows aligns with the greater DMI, resulting in higher energy intake and less energy expenditure for walking and grazing. Based on our estimations using NASEM (2021) equations, the energy required for activity (grazing and walking) was on average ~10% of the maintenance requirements. The DMI of CB-TMR cows is an observed value on a pen-basis. When applying the energy balance back calculation using NASEM (2021) equations to estimate DMI, we found that the calculated DMI of CB-TMR cows was, on average, only 1.6% lower than the observed DMI. This suggests the basal NASEM (2021) equations, at least for confined treatment, apply to the cows and environment in the present experiment.

Regarding milk composition, differences were found only in lactose content, which was higher for CB-TMR cows compared with CB-GRZ cows, whereas neither differed from OD-GRZ cows. Lactose levels are influenced by glucose availability in the mammary gland (Rigout et al., 2003). Ruminal propionate is the predominant precursor for glucose synthesis (Drackley et al., 2001), which, in turn, is positively related to the amount of concentrate consumed by cows, and, in particular, by its starch content (Bannink et al., 2006). This may explain why the confined cows had the highest lactose content in their milk, as the forage-to-concentrate ratio was 40:60 for confined cows and 63:37 for mixed feeding cows, and the confined cows consumed a diet with 1.6 times more starch than the mixed feeding cows.

With respect to 305 d solids yield, the magnitude of the difference between confined and mixed feeding cows was greater for the spring- than for the autumn-calving season. The difference in lactation performance by calving season is likely related to the amount of grazed pasture, supplemental feed offered, and weather characteristics. García and Holmes (2001) reported higher yields of total milk solids for autumn-calved cows, partly due to longer lactations but also because of higher daily yields in mid and late lactation. However, there are also reports of the calving season not affecting lactation performance, with cows consuming on average 7.5 kg of concentrate/d (White et al., 2002). In contrast, Spaans et al. (2019) compared the planned start of the calving season in January, April, July, and October in New Zealand with a system with little to no supplementation in a temperate, warm-summer climate zone (Beck et al., 2018). The study reported that cows calving in July had the highest milk production and required numerically the least supplementation (3 kg DM of pasture silage/cow per day only during the late lactation). This outcome was attributed to greater pasture growth in spring aligning with the early to mid-lactation period when DMI requirements are elevated, associated with the fact that fresh pasture in spring is superior in digestibility and AA composition than conserved feeds. It is well-known that calving date in pasture-based systems is a key factor influencing pasture utilization by affecting the alignment between feed demand and pasture supply (García and Holmes, 1999; McCarthy et al., 2013), but the differences in pasture utilization are attenuated when the same grazing and feeding management decisions are applied (García and Holmes, 2005; Spaans et al., 2019). Beyond pasture utilization, in hot-summer climate regions such as in the present study, spring-calving mixed feeding cows are more exposed to environmental stressors, particularly heat stress, as they reach mid-lactation during the peak summer months. In contrast, autumn-calving cows experience summer during late lactation, which may reduce the effect of heat stress on production and welfare.

Feed conversion efficiency is a key factor in determining dairy cow performance. In this study, confined cows had a greater feed efficiency compared with mixed feeding cows. It is widely accepted that as DMI, and consequently milk production, increases, feed efficiency improves because a greater proportion of the feed is partitioned toward milk production rather than maintenance (the "dilution of maintenance" concept; Beever and Doyle, 2007). Although confined cows expend less energy on physical activity compared with mixed feeding cows, the amount of energy for activity was only 10% of maintenance energy requirements, according to NASEM (2021) calculations, and the effect on feed efficiency differences is therefore limited. Additionally, mixed feeding cows often do not reach their maximal DMI potential (Fajardo et al., 2015). For example, Bargo et al. (2002) reported a feed conversion efficiency of 1.28 for full TMR-fed cows and 1.14 for mixed feeding cows (30% pasture plus 61% TMR and 9% concentrate) in a 21-wk mid-lactation trial. The DMI was 26.7 and 25.2 kg/cow per day, and the ECM production was 34.2 and 28.8 kg/cow per day for full TMR and mixed feeding diet, respectively. In the present study, mixed feeding cows consumed an average of 37% pasture and 63% MR, with a feed conversion efficiency of 1.25 kg ECM/kg of DM. The lower feed conversion efficiency in mixed feeding cows is only slightly related to differences in dietary NEL content, as the NEL content of the mixed feeding diet was only 2% lower than that of the TMR. In contrast, mixed feeding cows consumed 33% less concentrates/kg of ECM than the confined cows, raising an important consideration regarding the use of human-edible grains for animal feeding. Following Wilkinson (2011), who defined human-edible proportions of feed ingredients, mixed feeding cows had a greater return on human-edible input (8.0 kg ECM/kg human-edible DM consumed) compared with confined cows (5.6 kg ECM/kg human-edible DM consumed).

The higher NUE observed in confined, autumn-calved cows reflects better N utilization, possibly related to a better overall feed efficiency and an improved nutritional balance provided by the TMR. These cows showed both a numerically lower CP content and a higher energy concentration in their diet, factors that have been associated with improved NUE in dairy cattle (Dijkstra et al., 2013). In contrast, the mixed feeding cows generally exhibited lower NUE. This outcome is consistent with studies that highlight the challenges of maintaining high NUE in grazing systems, where variability in pasture quality, N availability, and intake can lead to greater N losses through urine and feces, reducing overall N efficiency (Castillo et al., 2000). Another important aspect to consider is the imbalance between N and energy substrates in the rumen that may occur with the discontinuous feeding patterns typical of mixed feeding cows (Chilibroste et al., 1997). This imbalance can result in N loss in the rumen due to a mismatch between the degradation of N-substrates and the utilization of available N by microbes, leading to elevated rumen ammonia concentrations (Dijkstra et al., 2013).

The observed differences in BCS between mixed feeding and confined cows suggest a greater energy balance for the confined cows. The TMR diet was more glucogenic because of its higher starch content, resulting in a less severe negative energy balance (van Knegsel et al., 2007). In the spring-calving season, the BCS at calving was lower than in the autumn-calving season and below recommended levels (Roche et al., 2009). Despite higher energy intake and an earlier BCS nadir in CB-TMR cows compared with mixed feeding cows, the proportion of cows with a corpus luteum within the first 60 DIM was lower, indicating poorer reproductive function in confined cows. This suggests that confined cows prioritized milk production over reproduction, as increased nutrient availability primarily supports milk yield rather than reproductive performance (Horan et al., 2005). Higher DMI in confined cows may have increased liver blood flow and the metabolism of progesterone and estradiol (Sangsritavong et al., 2002), delaying ovarian cyclicity compared with pasture-based cows (Mee, 2012). This delay is critical for pasture-based seasonal calving systems, where resuming ovarian cyclicity and conceiving within 90 DIM is essential for aligning peak energy demands with pasture growth.

Economic Evaluation

The gross income, economic costs, and operating profit of the AVG-UY were lower than those of the experimental systems (CB-TMR, CB-GRZ, and OD-GRZ), whereas its return on assets was higher than CB-TMR and CB-GRZ but lower than OD-GRZ. Dairy systems in Uruguay rely heavily on pasture-based feeding with varying levels of supplementation and infrastructure investments (Fariña and Chilibroste, 2019). Pedemonte et al. (2024) analyzed the diversity of Uruguayan dairy farm systems, identifying different typologies based on intensification levels, participation of pasture in the diet, supplementation strategies, and productivity per hectare. Their findings highlight that less intensive farms, which rely mostly on pasture with minimal supplementation, tend to have lower profitability but not due to the use of pasture itself but because of low milk production per hectare and suboptimal pasture utilization, which limit their ability to absorb cost fluctuations. Conversely, farms with higher stocking rates and greater pasture intake per hectare achieved superior economic performance, particularly when strategic supplementation was used to balance seasonal variations in pasture availability. Similarly, Chilibroste et al. (2024) found that dairy farms that successfully increased productivity over time did so primarily by increasing home-grown forage consumption and stocking rates rather than relying solely on purchased feed. Their analysis of long-term dairy sector trends in Uruguay showed that higher pasture utilization, complemented by supplementation, led to greater efficiency and economic resilience. These findings reinforce the idea that profitability improvements in pasture-based dairy systems require intensification through better pasture management rather than simply increasing external feed inputs.

The AVG-UY, used as a baseline for economic analysis in this study, operates under a low-input, low-output model, reflecting the typical pasture-based dairy production strategy in Uruguay. Its gross income and economic costs were much lower (56%–79%) than the experimental treatments. The economic performance of AVG-UY aligns with findings from Baudracco et al. (2022), who evaluated strategies to double milk production per farm in Argentina. Their study found that intensifying pasture utilization and strategic supplementation improved financial outcomes, with intensified pasture-based systems achieving a higher return on assets than fully confined systems. Additionally, Baudracco et al. (2022) emphasized the financial risks associated with capital-intensive confinement systems, particularly under volatile milk prices and climate variability. This aligns with our finding that CB-TMR had the highest gross income and asset value but a relatively low return on assets, highlighting the challenge of recovering large infrastructure investments. In contrast, OD-GRZ achieved high profitability due to lower infrastructure costs, suggesting that improvements in grazing management and supplementation strategies could enhance economic outcomes without requiring full confinement. These findings are particularly relevant for countries such as Uruguay, where low-input farms face profitability constraints unless productivity per hectare is increased through better pasture management and supplementation strategies, as demonstrated by Pedemonte et al. (2024) and Chilibroste et al. (2024).

There were no differences in income over feed costs across treatments. Operating profit was similar between CB-TMR and CB-GRZ, whereas OD-GRZ had double the operating profit of these systems. Despite the similar operating profit between CB-TMR and CB-GRZ, they achieved this in different ways. The CB-TMR had both 2 times the gross income and costs of CB-GRZ, mainly due to higher concentrate use in the TMR diet. The return on assets for OD-GRZ was ~4 times that of CB-TMR, primarily due to lower infrastructure costs of OD-GRZ. In contrast, CB-GRZ matched OD-GRZ in productivity but required more costly infrastructure, resulting in the lowest operating profit and a return on assets similar to CB-TMR. To improve the viability of a CB infrastructure in a mixed feeding system, higher individual milk production or alternating its use among different groups of cows, or both, could maximize the use of the infrastructure and spread associated costs over a larger number of animals. Although CB-GRZ cows used the CB only half the day, a standard full-day space allocation per cow was maintained. Despite the previously discussed potential limitations of the OD-GRZ infrastructure, particularly under heat stress conditions, it remains an economically viable option for farmers, as no adverse effects on overall productive performance were observed. Future research should explore the long-term financial resilience of these models and evaluate strategies for improving productivity per hectare while maintaining cost efficiency.

Final Remarks

Confined cows outperformed mixed feeding cows in milk yield and solids production, driven by increased DMI, a more stable energy and nutrient supply, and improved feed efficiency, which helped maintain body reserves throughout lactation. The higher NUE reflected a more balanced energy-to-protein ratio, whereas mixed feeding cows faced greater variability in nutrient supply from pasture, leading to higher N losses. Despite lower overall production, mixed feeding cows maintained adequate milk composition and converted human-edible feed into milk more efficiently, aligning with sustainability goals. Seasonal differences in performance, particularly lower milk solids yield in spring-calving OD-GRZ cows, suggest that adverse environmental factors, especially heat stress, influenced production performance. This highlights potential challenges as extreme weather events may become more frequent.

Integrating these findings highlights the trade-offs between strategies. Confined cows achieved higher production and feed efficiency, whereas mixed feeding cows demonstrated a more sustainable nutrient use with lower infrastructure costs. Economic evaluation favored OD-GRZ as the most profitable system, despite its lower individual milk production. These results underscore the importance of tailoring dairy management strategies to balance productivity, resource use, and sustainability.

CONCLUSIONS

Under the conditions of this study, housing in either a CB or an OD during nongrazing periods did not significantly affect overall productive performance of cows in the mixed feeding system. Confined cows produced more milk than mixed feeding cows, regardless of calving season. Performance differences between mixed feeding and confined cows were more pronounced in spring-calving than in autumn-calving cows. The economic performance was higher for mixed feeding cows housed in the OD.

NOTES

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Nonstandard abbreviations used: AVG-UY = average Uruguayan dairy farm; CB = compost barn; CB-GRZ = CB housing with one grazing session plus MR supplementation; CB-TMR = CB-confined cows fed a TMR ad libitum; GRZ = one-session grazing supplemented with MR; MR = mixed ration; NUE = feed nitrogen use efficiency; OD = outdoor soil-bedded pens with shade structures; OD-GRZ = OD housing with GRZ; RPM = rising plate meter; THI = temperature humidity index.

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APPENDIX

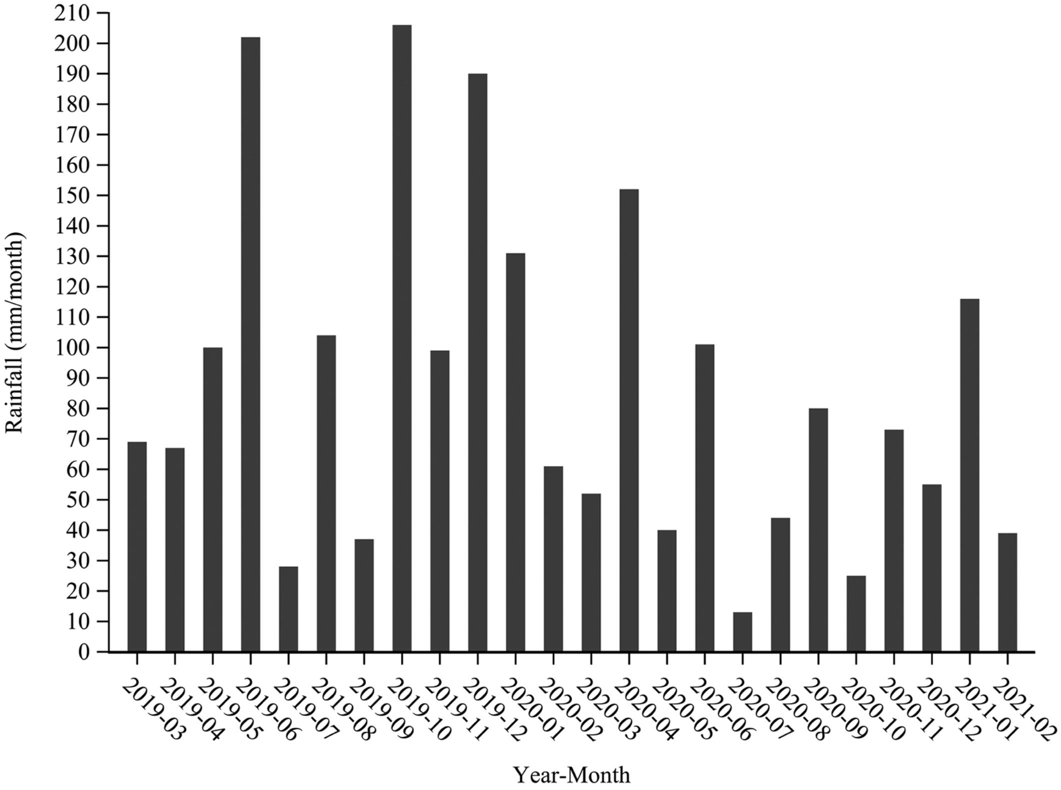

Figure A1. Monthly rainfall during the whole experimental period (March 2019–February 2021) at the research site. There were no records available for March 14 and 15, 2019, and from August 2 to 8, 2019, due to technical problems at the meteorological station.

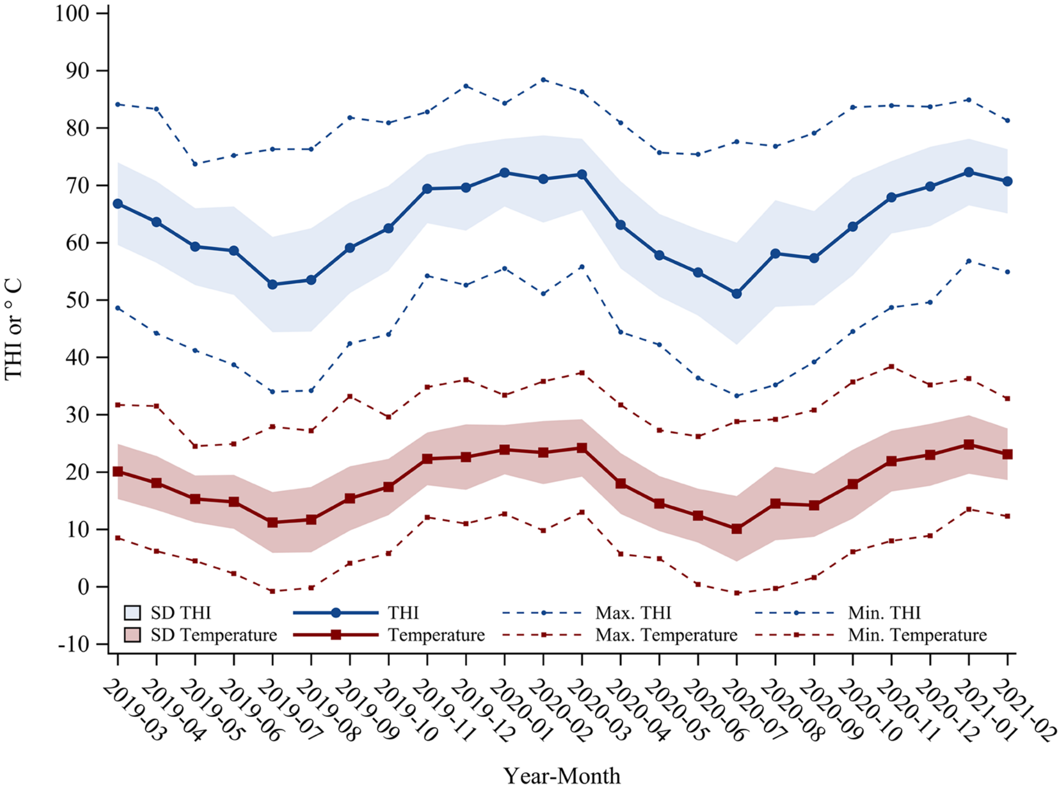

Figure A2. Monthly average, minimum, and maximum air temperature and humidity index (THI) during the whole experimental period (March 2019–February 2021) at the research site. There were no records available for March 14 and 15, 2019, and from August 2 to 8, 2019, due to technical problems at the meteorological station.

Table A1. Investments and annual costs (in US$) used in the simulations for the economic analysis of compost barn confinement with TMR (CB-TMR), CB housing with grazing and MR supplementation (CB-GRZ), and outdoor housing with GRZ (OD-GRZ)[1]

	Treatment			
Investment[2] and cost	CB-TMR	CB-GRZ	OD-GRZ	AVG-UY
Total investment, US$	1,619,151	932,245	315,413	353,481
Housing infrastructure	983,500	658,000	0	0
Dairy herd	293,955	106,585	106,585	135,370
Feed mixer	120,484	31,984	31,984	80,816
Milking parlor	49,843	23,197	23,197	68,614
Roughage reserved[3]	30,640	17,821	17,821	0
Bulk tank	81,420	31,924	31,924	20,355
Manure management	25,140	11,700	11,700	6,401
Milking parlor holding area	15,529	7,227	7,227	9,525
Milking machine	11,000	4,125	4,125	11,000
Aerial silos	7,640	7,640	7,640	412
Feeding area	0	0	41,168	0
Cow tracks	0	17,655	17,655	8,010
Water system at pasture	0	14,387	14,387	0
Others	0	0	0	12,978
Annual costs, US$/ha	5,278	2,669	2,473	1,039
Concentrates for adult cows	2,659	928	928	208
Supplements for heifer rearing	336	225	225	0
Paid labor	339	247	247	126
Improvements depreciation	275	200	57	22
Machinery hiring	217	156	156	100
Health, hygiene, insemination	205	137	137	51
Fertilizers	143	45	45	67
Imputed family labor	138	138	138	138
Substrate CB bed	135	61	0	0
Freight	124	51	51	20
Electricity	100	57	57	26
Taxes	99	58	78	26
Facilities repairs and maintenance	77	74	62	22
Administration	69	44	44	27
Seeds	69	47	47	51
Machinery repairs and maintenance	78	49	49	31
Machinery depreciation	77	49	49	29
Fuel	53	34	34	33
Herbicides	37	26	26	28
Technical assistance	25	25	25	18
Polyethylene for silos	23	18	18	16

[1] The simulations considered the necessary investments and costs to achieve the targeted milk production outcomes from experimental results, using the average Uruguayan dairy farm (AVG-UY; INALE, 2014) as a baseline.

[2] Investments have been included in annual costs by depreciation. For the AVG-UY, the values correspond to the actual infrastructure value of the farm, which was used as a baseline to scale up the experimental treatments.

[3] The roughage necessary to cover half of the requirement for 1 yr as a form of insurance in cases of adverse weather conditions.

Table A2. Average monthly rainfall during the 2 yr of study and last 15-yr average (2002–2017) at the research site

	Rainfall, mm/month		
Month	Average 2-yr of study[1]	Average last 15-yr	Difference (Study vs. last 15-yr)
January	124	133	−10
February	50	217	−167
March	61	151	−90
April	110	172	−63
May	70	123	−53
June	152	54	98
July	21	67	−46
August	74	109	−34
September	59	104	−45
October	115	142	−27
November	86	132	−46
December	123	140	−17
Total	1,043	1,543	−501

[1] There were no records available for March 14 and 15, 2019, and from August 2 to 8, 2019, due to technical problems at the meteorological station.

Table A3. Estimated milk yield parameters from the Wilmink exponential function[1] for compost barn confinement with TMR (CB-TMR), CB housing with grazing and MR supplementation (CB-GRZ), and outdoor housing with GRZ (OD-GRZ) during whole lactation with autumn- or spring-calving season

	Calving season (CS) and Treatments (TRT)							Probability		
	Autumn			Spring						
Item	CB-TMR	CB-GRZ	OD-GRZ	CB-TMR	CB-GRZ	OD-GRZ	SEM[2]	TRT	CS	TRT × CS
Parameter										
a	43.6^{AB}	43.8^{AB}	41.1^{B}	51.1^{A}	37.7^{B}	37.1^{B}	2.08	<0.001	0.706	0.003
b	−21.0	−24.3	−22.5	−21.6	−22.7	−18.8	2.07	0.127	0.466	0.521
c	−0.052	−0.080	−0.065	−0.085	−0.064	−0.064	0.0100	0.605	0.607	0.041
k	0.091^{AB}	0.048^{B}	0.047^{B}	0.071^{AB}	0.151^{A}	0.189^{A}	0.0264	0.779	<0.001	0.027

^{A,B}Means within a row with different capital letters differ ($P < 0.05$) between treatments and calving season.

[1] The Wilmink (1987) exponential function was fitted to daily milk yield data for individual lactations as $y_t = a + b \times e^{-kt} + c \times t$, where y_t represents the milk yield at day t of lactation, a is the initial milk yield after calving, b is the parameter determining ascending slope before the peak, c is the parameter determining descending slope after the peak, k is a factor related to the moment of peak yield, and e^{-kt} is an exponentially decreasing component.

[2] Average SEM is listed.

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Research article

Contrasting feeding management in the first 21 days *postpartum* in Holstein dairy cows: direct and residual milk responses

C. Rivoir[a,*], G.R. Mendina[b], M.L. Adrien[b], A. Meikle[c], P. Chilibroste[a]

[a] *Departamento de Producción Animal y Pasturas, Facultad de Agronomía, Universidad de la República, Paysandú, Uruguay*
[b] *Departamento de Ciencias Veterinarias y Agrarias, Facultad de Veterinaria, Universidad de la República, Paysandú, Uruguay*
[c] *Departamento de Clínicas y Hospital Veterinario, Facultad de Veterinaria, Universidad de la República, Uruguay*

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ABSTRACT

The transition period and its management affect how dairy cows face physiological challenges. Total mixed rations (**TMR**) have been shown as a strategy to address pasture-based system limitations and improve milk production, without consistent information about their use in short periods and residual responses. The study aimed to evaluate contrasting feeding management in the first 21 days *postpartum* on direct and residual milk production responses until 60 days in milk (**DIM**). Sixty-seven mixed parity Holstein dairy cows were used in a completely randomised block design in two treatments across two trials: MD-MD, cows fed a mixed diet (**MD**) with grazing and supplementation with mixed ration, from calving until 60 DIM, TMR-MD: confined cows with TMR provided *ad libitum* during the first 21 DIM in a compost-bedded pack barn and changed to MD at 22 until 60 DIM. During the first 21 DIM (direct response), cows fed TMR produced 11.7% more milk than those on MD-MD. Multiparous (**M**) TMR-MD obtained 18.6% higher milk yield than M MD-MD cows, but no significant differences were detected between primiparous (**P**) cows. After the switch at 22 DIM (residual response), no significant differences were detected between treatments in milk yield. However, the interaction between treatment and parity indicates that M TMR-MD cows exhibited 3.6% more milk than those in MD-MD, as a carryover effect. In contrast, P cows did not differ between treatments. In the first 21 DIM, TMR-MD cows achieved a higher fat concentration and protein yield than MD-MD. Fat yield tended to increase in TMR-MD compared to MD-MD, without treatment effect for lactose yield. In the residual period, TMR-MD cows had higher protein concentrations and tended towards higher fat concentrations than MD-MD cows. The interaction between treatments and parity showed that P TMR-MD cows had higher protein and exhibited a trend towards higher fat concentration than P MD-MD cows. The differential feeding management during the first 21 DIM did not generate differences in body condition score (**BCS**), between treatments or parities. In the residual period, TMR-MD cows achieved a higher BCS than MD-MD cows without differences between parities. No differences were found between treatments in grazing and ruminating time, reflecting a successful adaptation of TMR-MD cows. In conclusion, feeding TMR during the first 21 DIM is an effective strategy for increasing milk yield and achieving short-term carryover effects in M, but not in P cows.

Reader comments

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Implications

The transition period and its management impact dairy cows' physiological challenges. Total mixed rations can address pasture-based diet limitations and improve milk production. However, the impact of short-term total mixed rations on the overall performance of grazing systems remains unclear. Feeding a total mixed ration to multiparous cows during the first 21 days in milk, followed by a switch to a pasture-based diet, led to increased milk yield and positive carryover effects through the first 60 days of lactation. The switch did not lead to losses in body condition score,

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* Corresponding author.
E-mail address: crivoir@fagro.edu.uy (C. Rivoir).

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with successful grazing adaptation. This nutritional intervention does not improve productive performance in primiparous cows.

Specifications table

Subject	Nutrition
Type of data	Table, Figure.
How data were acquired	Milk yield was recorded individually by the milk measurement system GEA Dairy Plan C21 (Version 5.3). Milk samples for fat, total protein, and lactose were determined by infrared (MilkoScan FossElectric FT2®, Drachten, The Netherlands). Grazing and rumination time were measured with Boumatic® devices. Sward height was measured with the Sward Stick. Acid and neutral detergent fibres were analysed using an Ankom 200 Fiber Analyzer (Ankom Technology Corp.). Statistical analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA).
Data format	Processed, pretreated, and calculated data in Microsoft Excel and SAS.
Parameters for data collection	Data were collected under experimental conditions. Variables: milk production, lactose, protein, fat concentrations and yield, grazing and ruminating time, and body condition score.
Description of data collection	The experimental period was from 21 days precalving to 60 days in milk for both experiments. Before calving, cows were blocked according to lactation number, expected calving date, BW, body condition score, and randomly assigned to the treatment groups. Daily milk production recording, feed sample collections per week, weekly herbage mass, height and allowance recording, milk samples collection per 14 days (experiment 1) and 7 days (experiment 2), daily grazing and rumination recording, and body condition score every 15 days by the same person.
Data source location	Institution: Estación experimental Mario A. Cassinoni, Facultad de Agronomía, Universidad de la República. City: Paysandú Country: Uruguay Latitude: 32°23′8.58″S Longitude: 58° 3′18.87″W
Data accessibility	Repository name: https://zenodo.org/ Data identification number: https://doi.org/10.5281/zenodo.14030724
Related research article	None

Introduction

The transition period in dairy cows is arbitrarily defined as the 21 days before and after calving (Grummer et al., 1995) and is one of the most critical stages. It is characterised by the mobilisation of body reserves and a decline in dry matter intake (**DMI**), leading to negative energy balance and losses in body condition score (**BCS**) (Grant and Albright, 1995). In pasture-based systems, dairy cows face limitations in achieving high DMI and fully expressing their potential milk yield compared to cows fed a total mixed ration (**TMR**) (Kolver and Muller, 1998). In addition, grazing systems often struggle to consistently provide sufficient nourishment in quantity and quality throughout the year, resulting in a structural imbalance between pasture supply and animal demand (Chilibroste et al., 2011; Kay et al., 2015). This imbalance is particularly pronounced during early lactation, threatening productive performance and system profitability. Implementing optimal feeding strategies is essential for reducing the gap between nutrient requirements and intake, and for achieving desired milk yields while minimising the magnitude and duration of negative energy balance (Meikle et al., 2018) in early lactation.

To address the limitations of grazing systems while improving milk production, the use of mixed diets (**MD**), which combine grazing with the supplementation of a mixed ration (a balanced feed comprising silage and concentrate to complement the nutrients obtained from grazing), as well as TMR, has been proposed (Fajardo et al., 2015; Méndez et al., 2023). Feeding TMR increases milk yield by 38% (O'Neill et al., 2011) to 49% (Kolver and Muller, 1998) compared to cows under grazing without supplementation, and achieves 7–25% higher yields than cows on MD (Kennedy et al., 2015; Méndez et al., 2023). However, the response varies depending on parity, lactation stage, consumption, diet composition, pasture quality, and the energy demand associated with walking and grazing (Vibart et al., 2008). Feeding TMR in multiparous (**M**) cows in early lactation consistently enhances milk and solids yield compared to MD (Kolver and Muller 1998; Fajardo et al., 2015; Salado et al., 2018) due to an increased DMI and energy intake. In primiparous (**P**) cows, responses to different feeding strategies are inconsistent. Some studies report higher milk production with TMR compared to MD (Chilibroste et al., 2012), while others find no significant differences (Jasinsky et al., 2019). However, TMR has a positive impact on BCS, reproductive performance, and endocrine/metabolic profiles (Meikle et al., 2013b). Newly calved P cows under grazing conditions experienced a more severe negative energy balance compared to M cows (Meikle et al. 2004, Adrien et al. 2011) and faced difficulties adapting to grazing, reflected by low grazing activity and biting rates during pasture access (Chilibroste et al., 2012, Iqbal et al. 2022). Questions have arisen regarding the use of TMR in early lactation (Méndez et al., 2023), particularly concerning the direct and residual productivity responses, considering parity during the transition period. This highlights the need for a deeper understanding of how different feeding management during the transition period impacts animal performance.

Previous studies primarily report direct milk production responses when comparing TMR and pasture-based diets. According to a review (Jørgensen et al., 2016), only seven studies have explored the carryover effects of early lactation, focusing on concentrate levels and postgrazing height (Ganche et al., 2014). Studies have reported varied responses without considering parity as a factor, and none have made comparisons with TMR. The mechanisms underlying residual responses remain incompletely defined and operate at multiple levels (Jørgensen et al., 2016). When faced with dietary changes, cows can assess their environment and adapt to different feeding regimens by adjusting productive levels in response to ruminal and hepatic nutrient flow variations. This behavioural flexibility allows them to meet physiological needs (Delaby et al., 2009). Transitioning from TMR to pasture-based diets may further influence grazing behaviour, reflecting these adaptive responses.

To our knowledge, no previous research has exclusively focused on feeding during the transition period, considering direct and residual responses by parity and grazing adaptation. The hypotheses were as follows: (1) cows confined with TMR during the first 21 DIM (direct response), will increase milk and solids yield and BCS compared to an MD for P and M cows. (2) During the transition from TMR to MD, the differential milk production observed during the first 21 DIM will be sustained afterwards (residual effect). (3) Increasing grazing time will be one of the mechanisms to maintain milk production in cows switched from TMR to MD. Thus, an experiment was conducted to study the direct and residual productive responses and grazing adaptation during the initial 60 DIM of P and M Holstein dairy cows exposed to contrasting feeding management during the first 21 DIM.

Material and methods

Cows and experimental design

The study was carried out at the Experimental Research Station Dr. Mario A. Cassinoni of the Facultad de Agronomía, Universidad de la República (Paysandú, Uruguay; 32° S, 58° W). The study was replicated in two experiments based on the predominant calving seasons in Uruguay, autumn (experiment 1, from February to May 2021) and winter-spring (experiment 2, from June to October 2021) using a completely randomised block design. Before calving, cows were blocked according to lactation number, expected calving date, BW, BCS, and randomly assigned to the treatment groups. The treatments started immediately after calving and continued until 60 DIM, which comprised:

MD-MD: Cows fed a MD with grazing after a.m. milking (8 h access to paddock) and supplementation with mixed ration after p.m. milking, from calving until 60 DIM.

TMR-MD: Confined cows with TMR provided *ad libitum* during the first 21 DIM in a compost-bedded pack barn and switched to MD at 22 DIM and evaluated until 60 DIM.

Initially, 72 total Holstein dairy cows were allocated. Because of calving complications or serious illnesses (caesarean section, metritis, downer cow syndrome), 5 animals were removed from the experiment. This resulted in a final enrollment of 67 cows, equally distributed between two experiments (experiment 1: 20 M and 10P, experiment 2: 23 M and 14 P). Cows had a lactation number 2.0 ± 1.6 and 2.2 ± 1.4 (mean ± SE), BW 668 ± 89 and 622 ± 84 kg, BCS at calving 3.3 ± 0.3 and 3.3 ± 0.4 (scale 1 (skinny) to 5 (fat) (Ferguson et al., 1994), and calving date 11 April 2021 ± 20 days and 26 July 2021 ± 14 days for experiments 1 and 2, respectively.

All the cows had been exposed to pastures and had grazing experience as growing heifers and during their previous lactations. During the *prepartum* (21 days before parturition), the management was the same for all animals, and P cows were fed separately from M cows. They were offered a TMR diet composed of barley straw (experiments 1 and 2), corn (experiment 1), and sorghum (experiment 2) silage as a source of fibre and a *prepartum* commercial concentrate.

Feeding and grazing management

Cows in TMR-MD during the first 21 DIM were housed in a compost-bedded pack barn (13.2 m^2/cow) with automatic drinkers to ensure fresh water access, ventilation (fans), and sprinklers. The milk parlour was located 100 m from pens to minimise cow activity and long waiting periods during milking, which took place at 0500 and 1500 h. (for more details, see Méndez et al. 2023). Feeding access inside the barn was organised with galvanised sheet feeders along the front, providing 0.77 m/cow of space per cow on a concrete feeding area to reduce competition. The TMR was provided *ad libitum* once daily at 0800 h, ensuring 10% refusals (Brady et al., 2021). From DIM 22–60, TMR-MD cows joined the MD-MD treatment receiving the same feeding management and managed together.

Cows in MD-MD since calving and TMR-MD (22–60 DIM) had 7 h of daily access to weekly grazing plots after a.m. milking (from 0700–1400 h) and grazed together on annuals oats and raygrass pasture (*Avena sativa, Lolium multiflorum*), a second-year multispecies pasture with tall fescue (*Festuca arundinacea*), white clover (*Trifolium repens*) and birdsfoot trefoil (*Lotus corniculatus*), a third-year multispecies pasture with lucerne (*Medicago sativa*), and orchard grass (*Dactylis glomerata*), and biennial pasture with annual raygrass (*Lolium multiflorum*), and chicory (*Cichorium intybus*), located 1.7 km from the milking parlour with access to water in the pastureland.

The MD for MD-MD and TMR-MD (22–60 DIM) was formulated assuming that pasture availability was not limited (Table 1 and Table 2.), offering a herbage allowance three times higher than the expected DMI (25–30 kg DM/cow per day at ground level; Table 3). Grazing management followed a rotational system with weekly plot occupation. New plots were assigned based on herbage mass (kg DM/ha) and plant condition (e.g., number of leaves extended and/or nodes in lucerne) to ensure the target herbage allowance was consistently met (Table 3). After p.m. milking, cows were supplemented with a restrictive amount of mixed ration and remained in the same area overnight. In Experiment 1, cows were housed indoors in a compost-bedded pack barn under the previously specified conditions. In Experiment 2, cows remained in an outdoor soil-bedded yard with natural shade and water troughs.

Measures and samples analysis

Milk production was recorded individually, daily at each milking by the milk measurement system GEA Dairy Plan C21 (Version 5.3). Milk composition was evaluated from individual milk samples collected weekly in both experiments during two consecutive milkings. Fat, total protein, and lactose were determined by infrared (MilkoScan FossElectric FT2®, Drachten, The Netherlands). The energy in milk was calculated using the following equation:

$$\text{Energy in milk (Mcal/d)} = 0.0929 \times (\%\text{fat} + 0.0547 \times \%\text{protein} + 0.0395 \times \%\text{lactose}) \times \text{milk yield (L/d)}$$

Body condition score was visually recorded fortnightly in both experiments by the same observer using a 5-point scale with 0.25 increments (Ferguson et al., 1994).

To determine the appropriate paddock sizes for the target herbage allowance of 25–30 kg DM/cow per day at ground level, herbage mass (kg DM/ha) was estimated weekly in both experiments using a double-sampling technique adapted from Haydock and Shaw (1975). This method utilised a 3-point calibration scale and three replicates for each sampling level. Weekly, three replicate sets of 30 cm × 30 cm pasture squares were selected from each of the three sampling locations (low, medium, and high) within the grazing areas. In each square (totaling six), herbage was measured with a sward stick (Barthram, 1986), cut at ground level, collected, weighed, and sampled for DM content determination. Sward height was then measured in a zigzag pattern every five

Table 1
Ingredient composition (% of DM) of TMR and mixed ration fed to dairy cows.

	Experiment 1			Experiment 2		
Response[1]	Direct		Residual	Direct		Residual
Treatments[2]	MD-MD	TMR-MD	Both	MD-MD	TMR-MD	Both
Ingredients, % of DM						
Corn silage	34.0	36.6	43.9	15.0	44.3	16.4
Sorghum silage	–	–	–	27.6	–	24.3
Moha hay	2.1	6.3	0.8	1.7	4.6	–
Corn grain	17.9	22.0	15.5	19.1	17.4	21.0
Soybean meal	9.6	9.8	8.3	10.5	11.7	7.2
Canola meal	–	7.3	–	–	5.8	
Sunflower expeller	–	5.2	–	–	5.8	
Soybean hulls	–	9.8	–	–	7.7	
Wheat bran	24.6	–	21.3	22.7		24.1
Corn dried distillers' grain with soluble	9.6	–	8.3	–		3.1
Minerals and vitamins	2.2	2.5	1.9	3.7	2.5	3.9
Forage:concentrate ratio	36:64	43:57	45:55	44:56	49:51	41:59

Abbreviations: TMR = total mixed ration; MD = mixed diet; DIM = days in milk.

[1] Response: direct (weeks 1–3) and residual (weeks 4–9).

[2] Treatments: MD-MD = cows fed a MD from calving until 60 DIM, TMR-MD = confined cows fed with TMR provided *ad libitum* during the first 21 DIM and then moved to MD from 22 to 60 DIM.

Table 2
Chemical composition (% of DM) and offer of the TMR and mixed ration fed to dairy cows by treatment and response.

	Experiment 1			Experiment 2		
Response[1]	Direct		Residual	Direct		Residual
Treatments[2]	MD-MD	TMR-MD	both	MD-MD	TMR-MD	both
% of DM						
DM	58.3 ± 2.3	53.3 ± 4.4	60.1 ± 0.2	40.1 ± 2.1	50.2 ± 2.4	45 ± 4.1
CP	14.7 ± 2.3	17.0 ± 1.2	14.3 ± 0.2	14.3 ± 0.05	17.1 ± 0.3	14.3 ± 0.4
NDF	34.5 ± 1.1	35.5 ± 1.5	34.5 ± 0.3	39.2 ± 1.6	36.5 ± 1.8	40.0 ± 1.7
ADF	12.8 ± 1.8	17.1 ± 1.4	12.8 ± 0.3	17.6 ± 1.0	18.9 ± 1.6	17.4 ± 0.5
Ash	7.1 ± 0.3	7.9 ± 0.4	7.1 ± 0.2	9.1 ± 0.1	7.5 ± 0.4	9.9 ± 0.9
NEL (Mcal/kg DM)[3]	1.72 ± 0.03	1.65 ± 0.04	1.72 ± 0.01	1.64 ± 0.02	1.62 ± 0.05	1.65 ± 0.01
Allowance (kg DM/cow per day)[4]	12.6 ± 0.4	29.0 ± 1.2	14.1 ± 1.2	13,3 ± 0.5	29.9 ± 3.5	13.1 ± 0.8

Abbreviations: TMR = total mixed ration; MD = mixed diet; NEL = net energy of lactation; DIM = days in milk.

[1] Response: direct (weeks 1–3) and residual (weeks 4–9).

[2] Treatments: MD-MD = cows fed a MD from calving until 60 DIM; TMR-MD = confined cows fed with TMR provided *ad libitum* during the first 21 DIM and then moved to MD from 22 to 60 DIM.

[3] Estimated net energy of lactation calculated as 1.909 – (0.017 × ADF) according to NRC.

[4] Corresponds to the amount of mixed ration intake, as indicated by the empty feeders each morning for the MD-MD group during both the direct and residual periods, and for the TMR-MD group during the residual period.

Table 3
Chemical composition and characteristics of the pasture offered to dairy cows by to treatment and response.

	Experiment 1		Experiment 2	
Response[1]	Direct	Residual	Direct	Residual
Treatments[2]	MD-MD	Both	MD-MD	both
Herbage allowance, kg DM/cow per day	27.5 ± 4.3	24.4 ± 4	27.6 ± 0.6	27.1 ± 4.5
Pregrazing herbage mass, kg DM/ha	2 824 ± 682	2 054 ± 426	2 090 ± 535	2 128 ± 372
Postgrazing herbage mass, kg DM/ha	1 220 ± 635	1 166 ± 635	1 533 ± 714	1 481 ± 363
Pregrazing sward height, cm	17.7 ± 1.3	23.4 ± 1.3	24.2 ± 7.0	21.1 ± 4.1
Postgrazing sward height, cm	12.1 ± 0.8	14.3 ± 4.4	13.5 ± 1.3	14.5 ± 1.3
% of DM				
DM	25.3 ± 2.5	20.8 ± 4.6	23.7 ± 3.3	22.4 ± 4.1
CP	15.9 ± 4.0	21.3 ± 3.1	20.6 ± 4.3	18.7 ± 4.1
NDF	54.6 ± 8.4	39.3 ± 8.5	34.1 ± 7.1	36.1 ± 2.6
ADF	25.4 ± 2.7	17.2 ± 2.4	16.4 ± 3.0	17.6 ± 2.0
Ash	11.6 ± 1.4	12.1 ± 8.0	11.3 ± 1.4	11.3 ± 1.4
NEL (Mcal/kg DM)[3]	1.54 ± 0.06	1.73 ± 0.06	1.66 ± 0.03	1.64 ± 0.03

Abbreviations: MD = mixed diet; TMR = total mixed ration; NEL = net energy of lactation; DIM = days in milk.

[1] Response: direct (weeks 1–3) and residual (weeks 4–9).

[2] Treatments: MD-MD = cows fed a MD from calving until 60 DIM; TMR-MD = confined cows fed with TMR provided *ad libitum* during the first 21 DIM and then fed with MD from 22 to 60 DIM.

[3] NEL: Estimated net energy of lactation using (2.301 – (0.0289 × %ADF)) × 4.1868 × 0.239 (Acosta, 2004).

steps within the paddock using the sward stick. A linear regression relating sward height to DM mass from the calibration allowed for the determination of the mean herbage mass within the paddock.

Grazing behaviour (grazing and ruminating time during the grazing session) was measured individually, daily in both experiments in all the cows with Boumatic® devices fixed on a collar and placed around the cow's neck. These collars have been previously validated for Uruguayan dairy production systems (Fast et al., 2021).

The offer of TMR and mixed ration was recorded daily, in both experiments and representative samples of pasture, mixed ration, TMR, and components (silages, concentrate, and hay) were taken weekly in both experiments. The samples were weighed and oven-dried at 60 °C for 72 h to determine DM content. For pasture, the samples were obtained manually, simulating the residual sward height left by the cows. Samples were milled at 1 mm, and in each experiment, pooled and analysed monthly (TMR, mixed ration, and feedstuffs), except for pasture samples, which were analysed according to the different pastureland resources and paddocks. CP, NDF, and ADF were determined according to AOAC (2000). Total N for CP estimation used the Kjeldahl method of AOAC (1984), which involves sulfuric acid digestion with subsequent distillation and titration. NDF used amylase, and, as for ADF, an ANKOM200 Fiber Analyzer (ANKOM Tech. Corp., Fairport, NY, USA) was used.

Statistical analysis

Data were analysed in a randomised complete block design using the REPEATED statement in the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). For all variables, the cow, nested within the treatment and experiment, was used as the experimental unit. To study the impact of feeding management, the variables were analysed for the total response (weeks 1–9), direct response (weeks 1–3) during the application of contrasting feeding management, and residual response (weeks 4–9) to evaluate carryover effects when treatments were managed together. The models used were:

Milk production (total response, weeks 1–9):

$$Yij = \mu + T_i + P_k + WL_l + (T \times P \times WL)_{ikl} + B_j + \varepsilon_{ijkl}$$

where Yij is milk production, μ is the overall mean, T_i denotes the fixed effect of treatment (i = MD-MD or TMR-MD), P_k is the parity effect (k = M or P), WL_l denotes the week of lactation, $(T \times P \times WL)_{ijkl}$ is the fixed effect of the interaction, B_j refers to block as a random effect, and ε_{ijkl} denotes the residual error.

Milk production and composition (direct response weeks 1–3, residual response weeks 4–9):

$$Yij = \mu + T_i + P_k + (T \times P)_{ik} + B_j + \varepsilon_{ijk}$$

where Yij is milk production and all variables for milk composition, μ is the overall mean, T_i denotes the fixed effect of treatment (i = MD-MD or TMR-MD), P_k is the parity effect (k = M or P), $(T \times P)_{ijk}$ is the fixed effect of the interaction, B_j refers to block as a random effect and ε_{ijk} denotes the residual error.

Body condition score was analysed based on both the direct response during weeks 1–3 and the residual response from weeks 4 to 9. Additionally, two delta (Δ) values were assessed: the difference between BCS at calving and BCS at 21 dpp, and the difference between the average BCS during the direct response period and the residual period.

$$Yij = \mu + T_i + P_k + (T \times P)_{ik} + \mathrm{CO} + B_j + \varepsilon_{ijk}$$

where Yij is body condition score, μ is the overall mean, T_i denotes the fixed effect of treatment (i = MD-MD or TMR-MD), P_k is the parity effect (k = M or P), $(T \times P)_{ijk}$ is the fixed effect of the interaction, CO is the BCS at calving used as a covariate, B_j refers to block as a random effect and ε_{ijk} denotes the residual error.

Grazing behaviour (residual response weeks 4–9):

$$Yij = \mu + T_i + P_k + WL_l + (T \times P \times WL)_{ikl} + \mathrm{C} + B_j + \varepsilon_{ijkl}$$

where Yij is grazing and rumination time, μ is the overall mean, T_i denotes the fixed effect of treatment (i = MD-MD or TMR-MD), P_k is the parity effect (k = M or P), WL_l is the week of lactation. $(T \times P)_{ijk}$ and $(T \times P \times WL)_{ikl}$ is the fixed effect of the interaction, C is access time to the paddock day used as a covariate to appropriately account for differences across days, B_j refers to block as a random effect, and ε_{ijk} denotes the residual error.

The values reported are least-square means and SEs of least-square means. The model (co)variance structure was selected based on the smallest Bayesian information criterion value. Statistical significance was assumed at $P < 0.05$, and a tendency toward significance was assumed at $P \geq 0.05$ but <0.10. Results are shown as the mean ± SEM.

Results

Productive response

Overall response (1–9 weeks of lactation)

Cows fed a TMR in the first 21 DIM produced significantly more milk than cows in a pasture-based system (34.1 vs 32.9 L/d, P = 0.0057, TMR-MD and MD-MD, respectively). The triple interaction between treatment, parity, and week of lactation indicated that the evolution of milk production differed significantly (Fig. 1, P = 0.01). Specifically, M TMR-MD cows exhibited higher milk yield compared to M MD-MD cows at weeks 2 and 3. At week 2, M TMR-MD cows produced 39.2 L/d compared to 33.2 L/d for M MD-MD cows (P = 0.0004). At week 3, M TMR-MD cows peaked significantly higher, producing 41.0 L/d compared to 35.3 L/d for M MD-MD cows (P = 0.0001). The switch of M TMR-MD cows to the MD did not result in a significant decline in milk yield within the treatment (41.0 L/d at week 3 vs 39.6 L/d at week 4; P = 0.99). The residual response mainly occurred in the first 3 weeks after the switch, with M TMR-MD cows maintaining milk production. As lactation progressed, the milk yield curves of both treatments gradually converged (Fig. 1).

Feeding P cows with TMR after calving resulted in a numerical increase in milk production compared to those with MD, but the difference was not statistically significant. Primiparous cows peaked at 29.8 and 27.8 L/d at week 3 for TMR-MD and MD-MD, respectively (P = 1.0). The switch in P cows consuming TMR to MD did not result in statistically significant differences within the treatment (29.8 L/d at week 3 vs 28.5 L/d at week 4, P = 1.0), and the lactation curves evolved similarly between treatments (Fig. 1).

Direct response (0–3 weeks of lactation)

Cows fed TMR exhibited an 11% increase compared to those with MD (33.4 vs 29.9 L/d for TMR-MD and MD-MD, respectively, P = 0.006, Table 4). Additionally, the M cows produced significantly more milk than the P cows (36.4 vs 26.9 L/d, respectively, P < 0.0001). An interaction between treatment and parity was observed (P = 0.004), where M TMR-MD cows presented higher milk yield than M MD-MD (39.5 vs 33.3 L/d, P = 0.0003), while no significant differences were detected between P (27.2 and 26.6 L/d for TMR-MD and MD-MD, respectively, P = 0.98).

Feeding TMR affected fat concentration during the first 3 weeks (Table 4, P = 0.029) but not lactose and protein concentrations. Protein yield was higher and fat yield tended to increase in TMR-MD

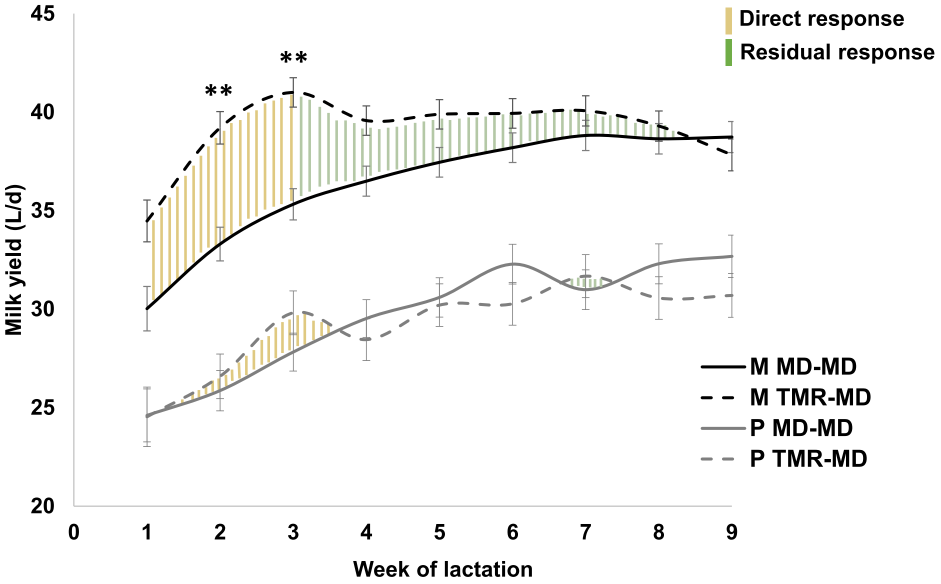

Fig. 1. Evolution of milk production in dairy cows under contrasting feeding management during the first 21 DIM (mixed diet (MD) and total mixed ration (TMR)) and by parity (multiparous (M) and primiparous (P)). Error bars represent the SE of the mean. Statistical significance is indicated by * ($P \leq 0.05$), and ** ($P \leq 0.01$) between treatments inside the parity in a particular week.

Table 4
Milk production, composition, and BCS in dairy cows under contrasting feeding management during the first 21 DIM, according to the direct and residual response.

Response[1]	Variable	Treatments[2]		SEM	*P*-value		
		MD-MD	TMR-MD		T	P	T × P
Direct	Milk yield, L/d	29.9[b]	33.4[a]	1.21	0.006	<0.0001	0.024
	Fat, %	3.59[b]	4.10[a]	0.22	0.029	0.705	0.864
	Fat, kg/d	1.11	1.33	0.12	0.074	0.002	0.274
	Protein, %	3.61	3.60	0.08	0.993	0.643	NE
	Protein, kg/d	1.19[b]	1.32[a]	0.06	0.034	0.085	NE
	Lactose, %	5.05	4.96	0.07	0.179	0.717	0.631
	Lactose, kg/d	1.53	1.55	0.10	0.795	<0.0001	0.042
	Energy in milk, Mcal/d	24.5[b]	28.6[a]	1.41	0.006	0.0002	NE
	BCS, mean	3.10	3.18	0.05	0.140	0.072	0.271
	ΔBCS (0 to 21 DIM)	−0.10	−0.02	0.05	0.149	0.088	0.293
	ΔBCS (21 to 60 DIM)	−0.23	−0.20	0.04	0.527	0.025	0.215
Residual	Milk yield, L/d	34.8	34.9	0.45	0.765	<0.0001	0.005
	Fat, %	3.51	3.76	0.14	0.071	0.170	0.020
	Fat, kg/d	1.22	1.24	0.07	0.747	0.0002	0.308
	Protein, %	3.26[b]	3.37[a]	0.05	0.025	0.005	<0.0001
	Protein, kg/d	1.09	1.12	0.05	0.477	<0.0001	0.066
	Lactose, %	5.13	5.12	0.05	0.804	0.0007	0.601
	Lactose, kg/d	1.71	1.72	0.08	0.857	<0.0001	0.650
	Energy in milk, Mcal/d	25.5	25.3	0.94	0.835	<0.0001	0.344
	BCS, mean	2.86	2.96	0.04	0.025	0.736	0.546

Abbreviations: TMR = total mixed ration; MD = mixed diet; T = treatment; P = parity; NE = no estimated; BCS = body condition score; DIM = days in milk.

[1] Response: direct (weeks 1–3) and residual (weeks 4–9).

[2] MD-MD: cows fed a MD from calving until 60 DIM, TMR-MD: confined cows fed with TMR provided *ad libitum* during the first 21 DIM and then fed with MD from 22 to 60 DIM.

[a,b] Within rows, mean values that do not share superscripts differ significantly from each other ($P < 0.05$).

compared to MD-MD without treatment effect on lactose yield. A tendency for higher protein yield ($P = 0.085$) in M than in P was also found. Cows in TMR-MD had higher energy in milk than MD-MD ($P = 0.006$) and M cows than P cows ($P = 0.0002$). The differential feeding management does not imply differences in BCS either between treatments (3.2 vs 3.1 for TMR-MD and MD-MD, respectively $P = 0.13$) or within parity.

Residual response (4–9 weeks of lactation)

No significant differences in milk production were detected between treatments (34.9 vs 34.8 L/d for TMR-MD and MD-MD, respectively; $P = 0.765$) after changing from TMR-MD to MD. However, there was a significant interaction between treatment and parity, indicating that M TMR-MD cows exhibited higher milk production than M MD-MD (39.5 vs 38.0 L/d, respectively, $P = 0.04$) which was not found for P cows (TMR-MD = 30.3 and MD-MD = 31.4 L/d, $P = 0.41$). Furthermore, similarly to the direct period, milk production differed according to parity (M = 38.7 and P = 30.9 L/d, $P < 0.0001$).

A trend was observed for fat concentration ($P = 0.071$) in TMR-MD cows, with an interaction between treatment and parity. A tendency for higher fat concentration in P TMR-MD than in P MD-MD

(4.02 vs 3.43% for P TMR-MD and P MD-MD, respectively, $P = 0.057$) was also observed. TMR-MD cows presented a higher protein concentration in milk than MD-MD cows ($P = 0.025$). In addition, an interaction between treatment and parity was observed for protein. This resulted in differences between P cows (3.54 vs 3.22% for TMR-MD and MD-MD, respectively, $P = 0.0007$) but not between M cows. For the same component, there was a significant parity effect (3.38 vs 3.24% for P and M, respectively, $P = 0.005$). For lactose, only the parity effect was detected as significant where P cows presented higher concentrations of lactose than M cows (5.20 vs 5.04% for M and P, respectively, $P = 0.0007$). Despite the differences in milk components, fat, protein, and lactose yields did not differ among treatments. For energy in milk, only the parity effect was detected as significant. TMR-MD cows achieved a higher BCS than MD-MD (3.0 vs 2.9, respectively, $P = 0.03$), without an effect of parity ($P = 0.18$) or the interaction between treatment and parity ($P = 0.54$). Body condition score change from calving to 21 DIM showed no treatment differences (Table 4), with a tendency for M cows to lose more BCS than P cows. Similarly, BCS change between direct and residual periods was consistent across treatments (Table 4), but M cows experienced a significantly greater BCS loss than P cows (−0.27 vs −0.16, respectively, $P = 0.02$).

Grazing and rumination evolution

Cows that started grazing immediately after calving (MD-MD), regardless of parity, showed an increase in grazing time as DIM progressed (Fig. 2). In M MD-MD cows, the highest increase was at week 5, rising from 44% in the first week to 52% of paddock access time (533 ± 70 min/d). Primiparous MD-MD cows showed the highest increase at week 6, rising from 43% in the first week to 51% of paddock access time at week 6. Rumination time increased in both groups from calving to week 3 followed by stabilisation as lactation progressed.

When TMR-MD cows started grazing (weeks 4–9) and treatments were managed together, no differences were found between treatments in grazing (TMR-MD: 254 vs MD-MD: 261 min/d, $P = 0.186$) or rumination time (TMR-MD: 128 vs MD-MD: 125 min/d, $P = 0.406$), representing 48 and 24% of paddock access time, respectively. A parity effect was detected for both variables: M cows spent more time grazing (M: 265 vs P: 250 min/d, $P = 0.0034$) and ruminating (M: 132 vs P: 123 min/d, $P = 0.005$) compared to P cows. A significant interaction between treatment and parity was observed for grazing time ($P = 0.05$) but not for rumination time ($P = 0.66$). Differences were found between treatments and parities (M MD-MD: 263 min/d vs P TMR-MD: 243 min/d, $P = 0.01$) and within TMR-MD (M TMR-MD: 266 min/d vs P TMR-MD: 243 min/d, $P = 0.004$). The triple interaction was non-significant for grazing time ($P = 0.144$), but it was significant for rumination time ($P = 0.002$); however, no differences were detected between treatments or within parity groups at any week.

Author's point of views

Direct response (0–3 weeks of lactation)

The increased milk yield in M cows aligns with previous studies comparing TMR and MD feeding during early lactation (Fajardo et al., 2015), mainly due to higher DMI and lower energy expenditure (Kolver and Muller, 1998). In the present study, M cows fed TMR may have achieved higher DMI due to the *ad libitum* offer and confined infrastructure, compared to MD cows. The optimal NDF content of the TMR, which enhances DMI (Mertens, 1994), may have further contributed to higher energy intake (Salado et al., 2020). Moreover, the lack of energy expenditure from walking, searching, and grazing (Bargo et al., 2002) likely contributed to redirecting energy from maintenance toward milk production. For cows with a pasture-based diet, factors such as suboptimal rumen fermentation (Bargo et al., 2002), grazing time, and bite rate may limit nutrient intake and thus contribute to explain the difference in milk yield compared to cows consuming TMR (Kolver and Muller 1998). This, combined with the onset of lactation, suggests a behavioural adaptation process in grazing. The gradual increase in grazing time during the postpartum period is associated with the rise in herbage intake and total DMI (Bossen et al. 2009).

Studies comparing TMR and MD typically evaluate longer periods, ranging from calving to 60 DIM (Fajardo et al., 2015) or even across full lactation (Salado et al., 2020; Méndez et al., 2023). Only 2 studies have focused on differential feeding management in the first 4 weeks *postpartum*. Al Ibrahim et al. (2013) investigated the effects of differential diets over the first 100 DIM (TMR vs MD) during the first 21 DIM. They found that confined cows had a higher DMI (approximately 1 kg DM), but there were no significant differences in milk yield, primarily due to the restricted TMR offer (23 kg DM/d). However, their analysis examined the entire experimental period without distinguishing between direct and residual responses. When shorter-term dietary interventions are evaluated over long periods, the dilution effect can hide differences, making them statistically undetectable. Brady et al. (2021) evaluated similar dietary interventions in cows of mixed parity, comparing *ad libitum* TMR during the first 30 DIM with grazing and supplemented with 3 kg DM/d of concentrate, and found no significant differences in milk production. The authors attributed this lack of significance to small differences in DMI between treatments (0.5 kg DM/d), as well as low protein (15.5% DM basis) and starch levels (20.1% DM basis) in the TMR. The DMI values found in both studies range between 17 and 18 kg DM/d using the Holstein Friesian strain. In contrast, the present work used the North American strain, the same herd as Fajardo et al. (2015), which reported that cows fed TMR achieved a DMI of 26 kg DM/d at 4- and 5-weeks *postpartum*. The lower DMI in confined cows along with differences in ingredients, quality, and energy content might explain the lack of response observed in the previously mentioned studies. Nevertheless, the present study shows that offering M cows with *ad libitum* access can significantly improve milk production, suggesting as a strategic approach for achieving peak production in dairy systems.

In P cows, the lack of differences between treatments was unexpected. A 21-day full TMR feeding was anticipated to increase milk yield due to the adaptation challenges faced under grazing conditions at the beginning of lactation (Chilibroste et al., 2012), compared to M cows (Meikle et al., 2013a). Moreover, a recent study by Walsh et al. (2024) found that P cows had a lower increase in DMI compared to M cows (0.41 vs 0.49–0.55 kg DM/d) under grazing conditions. The authors indicated that a higher increase in DMI is expected when cows are fed *ad libitum*, suggesting that P cows may be more productive under high-feed conditions than in pasture-based systems. Meikle et al. (2013a,b) found higher milk production in P cows fed TMR compared to those on MD with different herbage allowance levels. Since similar responses were expected for P cows fed TMR, it is possible that the non-detection of differences was due to the higher level of supplementation in this study (MD-MD) compared to that reported by Meikle et al. (2013a,b). Other authors (Ceriani et al., 2018; Jasinsky et al., 2019) assessed two feeding strategies during early lactation in P cows: TMR vs MD (grazing with 70% of *ad libitum* TMR). They observed a trend of higher milk yield in the TMR group, with no significant differences in DMI between the feeding strategies. As supplementation levels increase, milk production in cows on MD improves and becomes similar to that of TMR cows, which makes it difficult to detect differences between the two feeding strategies. A study by Gaillard et al. (2016) evaluated high or low-energy diets

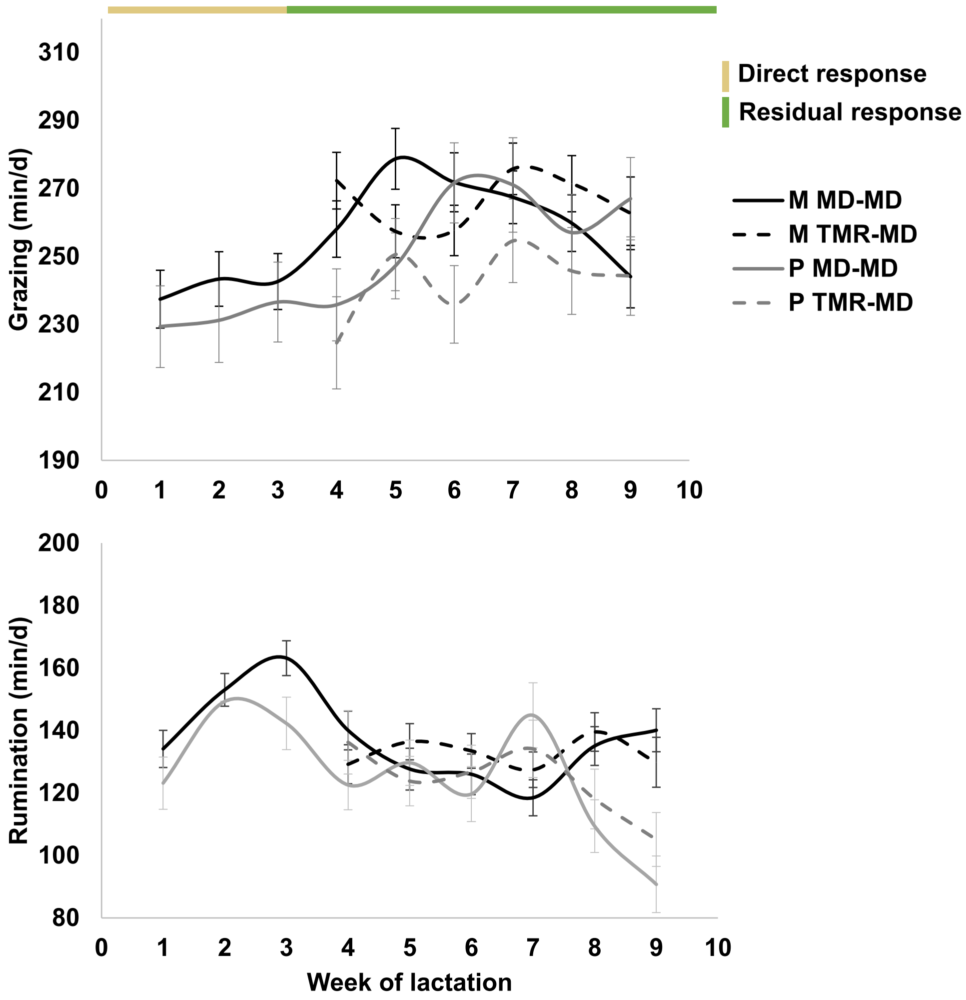

Fig. 2. Evolution of grazing and ruminating time at pasture in dairy cows under two different feeding management during the first 21 DIM (mixed diet (MD) and total mixed ration (TMR)) and by parity (multiparous (M) and primiparous (P)). Error bars represent the SE of the mean.

in the first 40 DIM in P cows and found that the extra energy was used for growth and body reserves, not for increased milk yield. In this experiment, differences in BCS were difficult to detect over a short period. It seems that a 21-day feeding TMR is insufficient for P cows, which require additional time to adapt to the new feeding regimen and daily routine to show a treatment response.

Residual response (4–9 weeks of lactation)

Switched M cows from TMR to MD allowed them to sustain higher production until 60 DIM, with the carryover response mainly in the first 3 weeks following the diet change. The causes may involve multiple mechanisms such as energy partitioning (Kennedy et al., 2007; Jørgensen et al., 2016), alveoli dynamics (Nørgaard et al., 2005; Ganche et al., 2014), and behavioural changes in eating patterns (Roche, 2007; Capuco and Choudhary, 2020). In M cows previously fed a high-energy diet during the first 40 DIM and then switched to a low-energy diet (Gaillard et al., 2016), higher DMI, energy intake, and milk production were observed in the first week after the change, indicating a short-term carryover effect. This study hypothesised that the residual response might be due to increased grazing time. However, the lack of differences in grazing and rumination time between treatments suggests that any potential increase in energy intake may result from other mechanisms, such as changes in pasture selectivity (Menegazzi et al., 2021). Mobilisation of body reserves does not appear to contribute to this response, as indicated by the higher BCS in TMR-MD cows.

Despite the abrupt dietary change from full TMR to a pasture-based diet, cows adapted rapidly and successfully to grazing, performing at levels to cows that had already been adapted since calving (MD-MD). In contrast, other studies have reported negative effects on animal performance when transitioning from TMR to a pasture-based system (Schären et al., 2016; Hartwiger et al., 2018) indicating a complex nutritional, behavioural, and metabolic adaptation. A gradual introduction to the MD system (grazing plus 4.5 kg DM of concentrate supplementation) from TMR resulted in a 12% decrease in milk yield in mixed-parity cows during mid-lactation (Hartwiger et al., 2018). Similar results were reported by (Schären et al., 2016) with a greater decrease (15–17%) due to the lower supplementation level (1.75 kg DM of concentrate/d). The magnitude of pre- and postfeeding and its duration are the most determining factors in residual productive responses (Jørgensen et al., 2016). As reported in previous studies, large changes in the quantities offered have a negative impact. In the present research, the shift from TMR to MD involved a high level of supplementation (13–14 kg DM/d) that represented 54–59% of the DMI (estimation based on energy balance, not reported) and optimal pasture conditions (2000–2100 kg DM/ha and 21–23 cm height). This combination minimised the impact on milk production and BCS (Fajardo et al., 2015). Over long analysis periods (+100 days), the residual response is diluted, as it primarily occurs in the short term, particularly during the initial weeks following a diet change. Most studies either covered extended residual periods where potential responses were undetectable (Kennedy et al., 2015) or did not evaluate residual responses (Al Ibrahim et al., 2013; Brady et al., 2021), where these responses are likely to occur. The findings of this study highlight the importance of analysing variables by period to capture residual responses and accurately estimate the total economic impact of a feeding strategy (Jørgensen et al., 2016).

Contrary to the findings in M cows, transitioning P cows to a MD at 22 DIM (TMR-MD) may not be advisable. Although P cows adapted well to grazing, performing at the same level as MD-MD cows, the transition to a new feeding strategy appears to have triggered tissue mobilisation. This is evidenced by higher milk concentrations of protein and fat (trending) compared to P cows that had already been adapted to grazing since calving (MD-MD). This category appears to be sensitive to feeding changes due to their ongoing adaptation to new management. Primiparous cows have additional requirements, as they are still growing, and the demands of lactation coincide with their growth requirements (Wathes et al., 2007). Further research is required to fully understand the mechanisms underlying these residual responses.

Conclusions

Implementing an *ad libitum* TMR strategy during the first 21 DIM in a confinement system, followed by a transition to a pasture-based diet, yielded parity-specific responses in productive performance. Multiparous cows exhibited immediate higher responses in milk yield with carryover effects, and adapted successfully to the pasture-based diet, suggesting a potential strategy for achieving peak production. In contrast, this dietary intervention did not enhance the productive performance of P cows, indicating that an extended TMR period may be needed to reveal their full productive potential. However, P cows in a pasture-based system with high supplementation successfully met their nutritional demands, supporting and optimising milk yield during early lactation. The lack of differences in BCS between dietary interventions in both parities underscores the need for further research on metabolic adaptation during this period, particularly in P cows. Additional studies on early DMI, grazing adaptation, and carryover effects may provide valuable insights for optimising transition management in pasture-based systems.

Peer Review Summary

Peer Review Summary for this article (https://doi.org/10.1016/j.anopes.2025.100092) can be found at the foot of the online page, in Appendix A.

Ethics approval

The experimental protocol was evaluated and approved by the Comisión Honoraria de Experimentación Animal (CHEA), Universidad de la República, Montevideo, Uruguay (Expe # 020300-000693-20).

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

Author ORCIDs

Catalina Rivoir: https://orcid.org/0000-0001-8269-0815.
G.R. Mendina: https://orcid.org/0000-0003-1311-4421.
Ana Meikle: https://orcid.org/0000-0002-0847-8629.
Maria de Lourdes Adrien: https://orcid.org/0000-0001-5914-1981.
Pablo Chilibroste: https://orcid.org/0000-0001-9579-9967.

Author contributions

CR, PC, AM, MLA, and GM: Conceptualisation, Methodology; CR, MLA, and GM: Investigation; CR and PC: curation; CR and PC: data analysis; CR: writing original draft; PC, AM and GM: reviewing; PC: supervision, project administration. All authors have read, reviewed, and agreed to the published version of the manuscript.

Declaration of interest

None.

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