Received: 14 November 2016 | Accepted: 8 March 2017

DOI: 10.1111/rda.12981

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

# Endometrial gene expression in primiparous dairy cows at the end of the voluntary waiting period is affected by nutrition: Total mixed ration vs increasing levels of herbage allowance

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**Funding information**
Agencia Nacional de Investigación e Innovación, Grant/Award Number: ANII #POS_2011_1_3577

**Contents**

The study postulated that differential nutritional management during the early lactation period would be reflected in endometrial expression of genes related to embryo growth at the end of the voluntary waiting period. Thus, the effect of the combined use of total mixed ration (TMR) and grazing under different herbage allowances during the first 75 days post-partum (DPP) on endometrial gene expression was evaluated in primiparous dairy cows. Cows were blocked by body weight, age and body condition score and randomly assigned to three grazing treatments: high (HA, 30 kg DM per cow per day), medium (MA, 15 kg DM per cow per day) and low (LA, 7.5 kg DM per cow per day) herbage allowance (mixed pasture, 2,600 kg DM per ha) plus 8 kg DM of supplement or TMR (55% forage, 45% concentrate) fed *ad libitum* (TMR) from calving to 75 DPP. At 57 DPP, cows were synchronized for oestrus (day 0, 68 DPP) and at day 7, endometrial biopsies were obtained. The nutritional treatment did not affect insulin, IGF-1 and leptin concentrations on days 0, 4 or 7. Expression of *IGF1*, *IGFBP3*, *IGFBP4*, *ADIPOR1* and *ADIPOR2* mRNA was significantly affected by the nutritional treatment. Endometrial *IGF1* and *IGFBP4* mRNA were twofold greater in TMR and HA than MA and LA cows. Expression of *IGFBP3* and *ADIPOR1* mRNAs was greater in TMR and HA than MA cows, but did not differ from LA cows. All groups had greater expression of *ADIPOR2* mRNA than MA cows. This study provided solid evidence of the importance of nutritional management during early lactation on uterine environment at the end of the voluntary waiting period. The greater expression of genes related to embryo growth and uterine function (IGF system, progesterone and adiponectin receptors) in cows fed diets maximizing energy intake suggests a favourable environment for embryonic growth, which may explain the improved reproductive performance of cows in good energy balance.

## 1 | INTRODUCTION

Reproductive performance of high-yielding dairy cows underwent a major decline during the last 50 years of the 20th century (1950 to 2000; Butler, Cummins, & Moore, 2015); more recently selecting for improved fertility has halted this continuous decline and has even started to improve (Norman, Wright, Hubbard, Miller, & Hutchison, 2009). The reproductive success after calving is limited by the resumption to ovarian cyclicity and the early embryo mortality which are both affected by the typical negative energy balance of early lactation (Santos, Thatcher, Chebel, Cerri, & Galvão, 2004). It is known that pregnancy establishment is vulnerable during the period of maternal

  

recognition of pregnancy and that 40% of pregnancy failures occurred during this period (Diskin, Murphy, & Sreenan, 2006), and the endometrium must produce an appropriate environment to stimulate embryo development and to allow implantation (Beltman et al., 2010).

The main regulators of uterine function are the oestrogens and progesterone that act via their specific nuclear receptors (ESR and PGR). In addition to the influence from the ovary, the post-partum uterus is exposed to the prevailing metabolic environment within the animal (Wathes et al., 2007). Several hormones such as growth hormone (GH)-IGF axis, leptin and adiponectin have been associated not only with post-partum ovarian cyclicity but also with uterine function (Meikle et al., 2004; Tabandeh, Hosseini, Saeb, Kafi, & Saeb, 2010). Reduced early post-partum insulin and IGF-1 concentrations due to feed restriction (20%) affected oviduct and endometrium expression of genes related with tissue remodelling processes in dairy cows at day 80 post-partum, which by impairing the normal functioning of the tract may explain the reduced reproductive performance of underfed cows (Valour et al., 2013). Indeed, recent data indicated that the mammalian endometrium appear to be a dynamic and reactive tissue which its compromised or suboptimal physiology can affect embryo development before implantation, with visible and sometimes severe consequences for the placentation process, foetal development and pregnancy outcome (Sandra et al., 2015). Many members of the GH-IGF axis are expressed locally within the endometrium, and their expression is altered by the stage of the oestrous cycle and pregnancy (Llewellyn, Fitzpatrick, Kenny, Patton, & Wathes, 2008; McCarthy, Roche, & Forde, 2011; Sosa et al., 2010). Moreover, in agreement with the known stimulatory effect of IGF-1 on both the embryo and the endometrium (Wathes, Reynolds, Robinson, & Stevenson, 1998), the endometrial expression of *IGF1* mRNA was positively associated with cow pregnancy at day 17 (Kirby, Thatcher, Collier, Simmen, & Lucy, 1996). Leptin and adiponectin—two of the better-studied adipokines—have been described to affect early embryonic development (porcine; Chappaz et al., 2008) directly or indirectly through their hormone receptors in the uterus (mice; Mirkin et al., 2005; Kim et al., 2011). In the bovine uterus, leptin receptor (*LEPR*) has been described in heifers (Sosa et al., 2010), but we did not find any reports on adiponectin receptors (ADIPOR).

It has been reported that grazing dairy cows do not get sufficient dry matter intake (DMI) to sustain the high milk production that could be achieved with their actual genetic potential (Kolver & Muller, 1998). Moreover, in mixed dairy systems, grazing management is key to sustain high milk yield and reduce production costs. Meikle, Adrien, Mattiauda, and Chilibroste (2013) showed greater milk production (20% more) when daily herbage allowance was increased from low (7.5 kg DM per cow per day) to medium (15 kg DM per cow per day), but no response was obtained when daily herbage allowance was increased to 30 kg DM per cow per day. Indeed, cows grazing medium herbage allowance sustained their high milk production by mobilizing energy reserves, which had a negative impact on the post-partum reinitiation of ovarian cyclicity. This led us to further hypothesize that the differential negative energy balance observed according to the different nutritional treatments, and consequent milk production, affected also uterine functionality at breeding time (end of the voluntary waiting period, at 68 DPP). Thus, this study aimed to analyse, in primiparous dairy cows at day 7 of oestrous cycle (critical period of early embryo loss; Diskin & Morris, 2008), changes on the endometrial gene expression induced by the combined use of total mixed ration and grazing under different sward herbage allowances. It was expected that cows fed diets that maximized nutrient and energy intake and reduced negative energy balance during early lactation would show an endometrial gene expression favourable to embryo growth.

## 2 | MATERIALS AND METHODS

The experiment was carried out at the EEMAC Research Station, School of Agronomy, Uruguay (30◦S, 53◦W). Animal procedures were approved by the Animal Experimentation Committee of University of Uruguay (UdelaR, Montevideo, Uruguay).

### 2.1 | Animals and experimental design

Primiparous Holstein dairy cows ($n$ = 44, 595 ± 41 kg of BW, 3.7 ± 0.3 of BCS) calving within 20 days during fall (May-June) were used. Cows were blocked by BW, age and BCS and randomly assigned to three grazing treatments ($n$ = 11 each): high (HA, 30 kg DM per cow per day), medium (MA, 15 kg DM per cow per day) and low (LA, 7.5 kg DM per cow per day) herbage allowance (mixed pasture, 2,600 kg DM per ha) plus 8 kg DM of TMR supplement or to TMR (55% forage, 45% concentrate) fed ad libitum (TMR) from calving to 75 days post-partum. The TMR supplement fed (i.e., 8 kg DM) once per day to the grazing treatments included, on a fresh weight basis, corn silage (10 kg) compound feed (4.8 kg) and grass hay (0.4 kg; roughage to concentrate ratio close to 55:45) and was formulated to meet maintenance metabolizable energy needs plus 8–10 L/d of milk (NRC 2001), leaving any difference in cow performance to the effect of grazing treatments.

Cows were milked twice a day (5:00 and 16:00), and milk yield was measured daily, and samples were obtained weekly for the analysis of protein, fat and lactose, and cow BCS was registered weekly (Edmonson, Lean, Weaver, Farver, & Webster, 1989). Data of BCS, milk yield, metabolite and hormone concentrations during the first 60 days of lactation and days to first ovulation have been published previously. At 60 days of lactation, milk yield and BCS were the greatest in TMR, the lowest in LA and intermediate in HA and MA cows which did not differ (29.5, 24.7, 23.2, 19.6 ± 0.4 L/d milk yield and 3.2, 2.8, 2.7, 2.4 ± 0.1 BCS in TMR, HA, MA and LA cows respectively; Meikle et al., 2013).

### 2.2 | Tissue collection and blood sample

At 57 days post-partum, cows were synchronized for oestrus. Oestrous synchronization was conducted in two groups of animals to homogenize days post-partum at the time of initiation of treatment. Hormonal treatment involved placing at day 9 (day 0 = oestrus), a natural

progesterone intravaginal device (Terapress ®) with an intramuscular injection of 2.5 ml of Fertagyl ® (0.25 mg of synthetic gonadotrophin hormone releasing hormone/cow). The implant was removed at day 2, and 2 ml of Enzaprost DC® (D-Cloprostenol/cow 0.15 mg) was injected intramuscularly, and patches were placed in the sacro-coccygeal region to aid in oestrous detection. Oestrous detection was conducted for 5 days after implant removal, by observing the animals twice daily (after each milking) for 40 min. At day 7 of the oestrous cycle (day 0 = oestrus) in a subsample of nine cows of each group endometrial biopsies ipsilateral to the corpus luteum were obtained as described by (Meikle et al., 2001). Endometrial samples were immediately frozen in liquid nitrogen and stored at −80°C until total RNA was isolated.

In the same cows in which endometrial biopsies were obtained, blood was collected at day 0 (oestrous or at 48 hr after to implant removed), 4 and 7 (day of biopsy) of the oestrous cycle by venipuncture of the coccygeal vein using Vacutest® tubes (8 ml, Vacutest Kima, Arzergrande, Italy) that contained clot activator gel. Samples were centrifuged (2,000× *g* for 15 min at 4°C) within 1 hr after collection, and serum was stored at −20°C until hormone analyses were performed.

## 2.3 | Isolation, purification of RNA, and real-time PCR

Although endometrial biopsies were collected from each animal, the amount of tissue was not enough for mRNA extraction for some of the samples, and therefore 7, 8, 8 and 6 samples were analysed for TMR, HA, MA and LA respectively.

Total RNA from uterine tissue was isolated using TRIzol® (Invitrogen, Life Technologies, Carlsbad, CA, USA), followed by precipitation with lithium chloride and by DNase treatment with a DNA-Free™ kit (Applied Biosystems/Ambion, Austin, TX, USA). Concentration of RNA was determined by measuring the absorbance at 260 nm (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technnologies, Wilmington, DE, USA), and purity and integrity of RNA isolates were assessed from 260/280 and 260/230 absorbance ratios (greater than 1.8) and by electrophoresis in 1% agarose gel. Isolated RNA was stored at −80°C until analysed by quantitative real-time PCR.

The SuperScript®III First-Strand Synthesis System kit (Invitrogen) was used to conduct the reverse transcription using random hexamers and 1 μg of total RNA as a template. The cDNA was stored at −20°C until its use in the real-time PCR. Primers (Table S1) to specifically amplify cDNA of target genes: *GHR*, *IGF1*, *IGF2*, *IGF1R*, *IGF binding proteins 1 to 6 (IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP4*, *IGFBP5*, *IGFBP6)*, insulin receptor (*INSR*), *LEPRB*, *ADIPOR1*, *ADIPOR2*, oestrogen and progesterone receptors (*ESR* and *PGR* respectively) and from endogenous controls: β-actin (*ACTB*), hypoxanthine phosphoribosyltransferase (*HPRT*) and ribosomal protein S9 (*RPS9*) were obtained from literature or specifically designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) based on bovine nucleotide sequences available from NCBI (http:www.ncbi.nlm.nih.gov/). Before use, primer product sizes (1% agarose gel separation) and sequence (Macrogen Inc., Seoul, Korea) were determined to ensure that primers produced the desired amplicons.

Real-time PCR reactions were performed in a total volume of 15 μl using KAPA SYBR® FAST Universal 2× qPCR Master Mix (Kapa Biosystems, inc. Woburn, MA, USA) according with Astessiano, Perez Clariget, Quintans, Soca, and Carriqury (2011) using the following standard amplification conditions: 10 min at 95°C and 40 cycles of 15 s at 95°C, 45 s at 60°C and 20 s at 72°C. Dissociation curves were run on all samples to detect primer dimers, contamination or presence of other amplicons. Each disc included a pool of total cDNA from bovine uterine samples analysed in triplicate to be used as the basis for the comparative expression results (exogenous control) and duplicate tubes of water (non-template control). Gene expression was measured by relative quantification (Pflaffl, 2009) to the exogenous control and normalized to the geometric mean expression of the endogenous control genes (*HPRT*, *ACTB* and *RPS9*). Expression stability of three selected housekeeping genes was evaluated using MS-Excel add-in Normfinder (MDL, Aarhus, Denmark), and the stability values obtained were 0.435, 0.219 and 0.295 for *HPRT*, *ACTB* and *RPS9* respectively. In addition, these three genes have been used before as an endogenous control in tissues from ruminants (Casal et al., 2014; De Brun et al., 2013), and they proved to be good housekeeping genes as their expression did not varied among treatments in this study. Amplification efficiencies or target and endogenous control genes were estimated by linear regression of a dilution cDNA curve (*n* = 5 dilutions, from 100 to 6.25 ng/tube). Intra- and inter-assay CV values were 2.2% and 4.3% respectively.

## 2.4 | Hormone analyses

Progesterone (P4) was determined to confirm ovulation after synchronization and was measured by a solid-phase radioimmunoassay (RIA) using a commercial kit (Coat and Count; Diagnostic Products, Los Angeles, CA, USA). All samples were analysed in a single assay; the sensitivity was 0.01 ng/ml; the intra-assay coefficients of variation (CV) were 10.6%. Ovulation was defined as increased P4 concentrations > 1 ng/ml on day 7 of the oestrous cycle, only cows that ovulated were included in the statistical analysis (6, 6, 5 and 4 animals per treatment TMR, HA, MA and LA respectively).

Concentrations of insulin and IGF-1 were measured using immunoradiometric assays (IRMA) with commercial kits (INS-IRMA; DIA Source Immuno Assays S.A., Belgium and IGF-I-RIACT Cis Bio International, GIF-SUR-YVETTE CEDEX, France, respectively) previously used in bovines by Astessiano et al. (2015). All samples were determined in a single assay for each hormone. For insulin, the assay detection limit was 1.1 μIU/ml, and intra-assay CV for control 1 (22 μIU/ml) and 2 (55 μIU/ml) were 5.3% and 8.6% respectively. For IGF-1, the assay detection limit was 0.29 ng/ml, and intra-assay CV for control 1 (41 ng/ml) and control 2 (521 ng/ml) was 5.54% and 5.34% respectively. Leptin concentrations were determined by a liquid-phase radioimmunoassay (RIA) using a commercial Multi-Species Leptin kit (RIA kit, Millipore, USA) previously reported in bovines (Pinotti & Rosi, 2006). The RIA had a sensitivity of 1.4 ng/ml. All samples were determined in the same assay, and the intra-assay CV for control 1 (4.2 ng/ml) and control 2 (18.8 ng/ml) was 7% and 5.2% respectively.

**TABLE 1** Hormone concentration in the beginning of the oestrous cycle in primiparous dairy cows in total mixed ration (TMR; *n* = 7), high (HA; *n* = 8), medium (MA; *n* = 8) and low (LA; *n* = 6) pasture allowances groups

| | Treatments[1] | | | | | *p*-value |
|---|---|---|---|---|---|---|
| | TMR | HA | MA | LA | *SE* | TREAT |
| Progesterone ng/ml | 2.60 | 2.16 | 1.85 | 1.47 | 1.23 | .78 |
| Insulin μIU/ml | 10.75 | 11.41 | 13.25 | 10.58 | 1.10 | .40 |
| IGF-I ng/ml | 78.52 | 87.82 | 80.61 | 90.52 | 8.70 | .75 |
| Leptin ng/ml | 4.08 | 4.74 | 4.52 | 4.78 | 1.21 | .79 |

[1]Treatments = cows were assigned to three grazing treatments: high (HA, 30 kg DM per cow per day), medium (MA, 15 kg DM per cow per day) and low (LA, 7.5 kg DM per cow per day) herbage allowance (mixed pasture, 2,600 kg DM per ha) plus 8 kg DM of supplement or to a TMR (55% forage, 45% concentrate) fed *ad libitum* (TMR) from calving to 75 days post-partum.
TREAT, Treatments.

### 2.5 | Statistical analyses

Data were analysed in a randomized block design using the SAS System programme (SAS Institute Inc., Cary, NC, USA). Univariate analyses were performed on all variables to identify outliers and inconsistencies and to verify normality of residuals. Data of hormone concentrations were analysed as repeated measures using the MIXED procedure with days as the repeated effect, the first-order autoregressive as the covariance structure and the Kenward–Rogers procedure to adjust the denominator degrees of freedom. The model included nutritional treatment, days, and their interaction, and block as random effect. As the interaction between treatment and days was not significant (except for progesterone concentrations), it was removed from the model. Uterine mRNA expressions were analysed using the same model without the repeated effect of day. Tukey–Kramer tests were conducted to analyse differences between groups ($\alpha = 0.05$). For all results, means were considered to differ when $p \leq .05$, and trends were identified when $.05 < p \leq .10$. Data are presented as least square means ± pooled standard errors.

## 3 | RESULTS

The nutritional treatment did not affect concentrations of P4, insulin, IGF-1 or leptin (Table 1).

The expression of *IGF1* mRNA was affected by the nutritional treatment ($p = .01$) and was twofold greater ($p < .05$) in TMR and HA cows than MA and LA cows (Figure 1a).

Although the effect of nutritional treatment on endometrial expression of *IGF1R* and *IGF2* mRNA did not reach significance (Table 2), when Tukey–Kramer tests were conducted, *IGF1R* mRNA was greater ($p < .05$) in HA than LA cows, while *IGF2* mRNA was greater ($p < .05$) in TMR than MA cows. Endometrial *IGFBP3* mRNA was greater ($p < .05$) in TMR and HA than MA cows (Figure 1b), but did not differ from LA cows, while *IGFBP4* mRNA was greater ($p < .05$) in TMR and HA than MA and LA cows (Figure 1c). Expression of *IGFBP5* mRNA was greater ($p < .05$) in TMR and HA than MA cows.

In addition, the expression of *ADIPOR1* and *ADIPOR2* mRNA was affected ($p \leq .04$) by the nutritional treatment. The *ADIPOR1* mRNA was greater ($p \leq .04$) in TMR and HA than MA cows, while LA cows did not differ from any group (Figure 1d). In contrast, the expression of *ADIPOR2* mRNA was greater ($p < .01$) in TMR, HA and LA than MA cows (Figure 1e).

Although the effect of nutritional treatment on *PGR* mRNA expression did not reach significance (Table 2), when Tukey–Kramer test was conducted, expression tended to be twofold greater ($p = .06$) in TMR and HA than LA cows (Table 2). The expression of other genes did not differ among treatments (Table 2).

When cows that did not ovulate were also included in the analysis and ovulation was added as fixed effect, similar results were obtained (data not shown). Moreover, mRNA expression of *PGR*, *IGF2* and *IGFBP5* showed trends to differ among treatments when all cows were considered in the analysis ($p = .11$, 0.13 and 0.09 respectively) and Tukey–Kramer test was as shown in table 2.

## 4 | DISCUSSION

The present study provides novel information on changes induced by the combined use of TMR and grazing different sward herbage allowances on endometrial gene expression at day 7 of the oestrous cycle (critical period of early embryo loss; Diskin & Morris, 2008) of dairy primiparous Holstein cows at the end of the voluntary waiting period. The uterine endometrium plays a central role in early conceptus-maternal communication for establishment and maintenance of pregnancy which involves dynamic changes in the uterine cell types tightly regulated by changes in steroid hormones, cytokines and growth factors and their receptors. Hence, the endometrium has to be considered a critical epigenetic contributor to the embryo trajectory from the very earliest stages of pregnancy (Sandra et al., 2015).

Although hormone concentrations did not differ in the early stages of oestrous cycle, relevant differences were detected in endometrial mRNA expression among treatments. All hormone concentrations were within the normal range, consistent with previous reports in dairy cows (Rhoads, Meyer, Lamberson, Keisler, & Lucy, 2008) and heifers (Killeen et al., 2014) during the same days of the oestrous cycle. It should be considered that lack of differences due to treatments on hormone concentrations in the present study may be explained, at least partially, by the low differences on energy balance between groups at beginning of the synchronization period (Meikle et al., 2013)

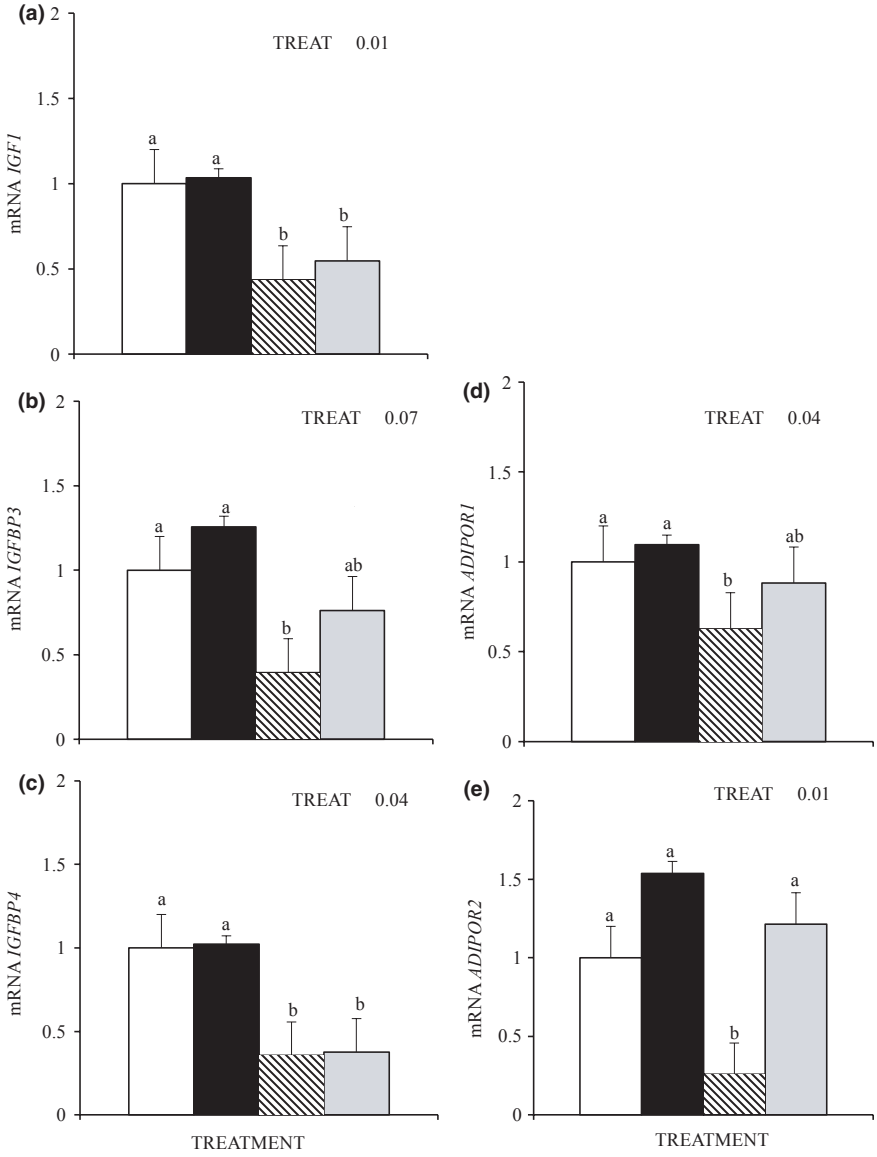


**FIGURE 1** Relative endometrial mRNA expression values for *IGF1* (a), *IGFBP3* (b), *IGFBP4* (c), *ADIPOR1* (d) and *ADIPOR2* (e) at day 7 of the oestrous cycle (day 0 = oestrus) in primiparous dairy cows in TMR (□), high (HA; ■), medium (MA; ▨) and low (LA; ▢) pasture allowances groups and in ovulated (□) and non-ovulated (■) cows. Within treatments: a vs b indicates significant differences ($p < .05$)

and by blood sampling frequency which was not optimal, as only three samples were collected.

The physiological status of the endometrium just before the moment of blastocyst hatching (uterus at day 7) is key in the development and future elongation of the embryo. In beef heifers, Forde et al. (2011) proposed that P4 mediated its effects predominantly via the endometrium at a time the embryo is transferred (day 7) will affect the future process of elongation. In the current study, the greater expression of *IGF1* mRNA in TMR and HA than MA and LA cows—for example animals with better nutritional status during early lactation—suggests a favourable uterine environment for embryo development. Indeed, P4 increases embryo growth and induces the secretion of IFNτ by acting on the endometrium to stimulate the production of a variety of embryotrophic factors (Geisert et al., 1991). Indeed, it has been shown that *IGF1* mRNA expression is regulated, primarily, by the action of P4 on the uterine endometrium (Satterfield et al., 2010). The expression of *IGF1* mRNA in the uterus was consistent with *PGR* mRNA expression, suggesting a favourable uterine environment for embryonic development in cows fed diets that maximized nutrient and energy intake during early lactation. The greater expression of these genes found in TMR and HA cows is consistent with the greater probability of post-partum ovarian cyclicity in these cows (Meikle et al., 2013).

Greater uterine *IGF1* mRNA (Meikle et al., 2001) and greater IGF-1 secretion into the uterine lumen (Wathes et al., 1998) around oestrus has been suggested to enhance the uterine environment on which the ruminant embryo is dependent during the pre-implantation period (Robinson, Fray, Wathes, Lamming, & Mann, 2006). Indeed, the endometrial expression of *IGF1* mRNA was positively associated with cow pregnancy at day 17 (Kirby et al., 1996). Further control of this growth factor is achieved via competitive binding to the IGFBPs, whose expression is regulated in a tissue-specific manner and influenced by animal metabolic status (Clemmons, 1998; Thissen, Ketelslegers, & Underwood, 1994). The lower endometrial expression of *IGF2* and *IGF1R* mRNA in MA and LA cows, respectively, is consistent with a poor endocrine and metabolic status previously reported in these animals (Meikle et al., 2013).

**TABLE 2** Relative endometrial mRNA expression values at day 7 of the oestrous cycle (day 0 = oestrus) in primiparous dairy cows in TMR ($n$ = 7), high (HA; $n$ = 8), medium (MA; $n$ = 8) and low (LA; $n$ = 6) pasture allowances groups cows

| | Treatments[1] | | | | | *p*-value |
|---|---|---|---|---|---|---|
| | TMR | HA | MA | LA | *SE* | TREAT |
| Gene | | | | | | |
| *PGR* | 1.00[a] | 1.09[a] | 0.64[ab] | 0.48[b] | 0.12 | .19 |
| *ESR* | 1.00 | 0.88 | 0.78 | 0.54 | 0.22 | .76 |
| *GHR* | 1.00 | 0.89 | 0.97 | 0.47 | 0.18 | .47 |
| *IGF1R* | 1.00[ab] | 1.47[a] | 0.82[ab] | 0.55[b] | 0.13 | .14 |
| *IGF2* | 1.00[a] | 0.55[ab] | 0.45[b] | 0.51[ab] | 0.17 | .18 |
| *LEPRB* | 1.00[a] | 0.72[a] | 0.81[a] | 0.30[b] | 0.12 | .27 |
| *INSR* | 1.00[a] | 1.39[a] | 0.51[b] | 0.97[ab] | 0.23 | .22 |
| *IGFBP1* | 1.00 | 1.79 | 2.35 | 2.32 | 0.02 | .56 |
| *IGFBP2* | 1.00 | 1.39 | 1.44 | 1.25 | 0.42 | .85 |
| *IGFBP5* | 1.00[a] | 1.03[a] | 0.47[b] | 0.57[ab] | 0.21 | .17 |
| *IGFBP6* | 1.00 | 0.99 | 0.76 | 0.84 | 0.27 | .50 |

Different letters between treatments: a vs b indicates significant differences ($p < .050$).
[1]Treatments = cows were assigned to three grazing treatments: high (HA, 30 kg DM per cow per day), medium (MA, 15 kg DM per cow per day) and low (LA, 7.5 kg DM per cow per day) herbage allowance (mixed pasture, 2,600 kg DM per ha) plus 8 kg DM of supplement or to a TMR (55% forage, 45% concentrate) fed *ad libitum* (TMR) from calving to 75 days post-partum.
TREAT, treatments.
Genes: *PGR*, progesterone receptor; *ESR*, oestrogen receptors; *GHR*, growth hormone receptor; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2*, insulin-like growth factor 2; *LEPRB*, leptin receptor; *INSR*, insulin receptor; *IGFBP1*, *IGFBP2*, *IGFBP5*, *IGFBP6*, IGF binding proteins 1, 2, 5 and 6.

In this study, endometrial expression of *IGFBP3*, *IGFBP4* and *IGFBP5* mRNA at day 7 of oestrous cycle was affected by nutritional treatments, being greater in TMR and HA than MA cows. The increased in *IGFBP3* mRNA supports a stimulatory role of this protein during early pregnancy as it has been reported that its expression responds to embryonic signals (Liu, Mele, Catz, Noyes, & Rosenwaks, 1995) and theorized that it may aid in the transportation of systemic IGF to its target site (Robinson et al., 2006). Several studies demonstrated that during development *IGF2* and *IGFBP4* mRNA are co-expressed with IGF2 in many tissues indicating a potential interaction between IGF2 and IGFBP4 in growth and a possibly reservoir of IGF within tissues (Ning, Schuller, Conover, & Pintar, 2008). Contradictory with our results, Wathes, Cheng, Fenwick, Fitzpatrick, and Patton (2011) showed that *IGFBP4* mRNA endometrial expression was greater in cows in severe than mild negative energy balance; however, it should be noted that this study was conducted at 14 days post-partum when uterine involution was not complete, animals were not cycling, and difference between groups on energy balance was greater than in our study. The increased expression of *IGFBP5* mRNA found in TMR and HA than MA cows is consistent with its stimulatory effects on IGF-1 action (Robinson et al., 2006). Overall, data of the IGF system suggest that endometrial expression is modulated according to the energy status.

Female reproductive functions are closely associated with nutritional status, and adiponectin seems to be an important factor linking the energy homoeostasis with reproductive function. The impact of adiponectin in the endometrium in productive species is almost unknown (Smolinska et al., 2014), and as far as we know this is the first description of adiponectin receptors in bovine uterus. In humans (Takemura et al., 2006) and rodent uterus (Mirkin et al., 2005), expression of adiponectin receptors (*ADIPOR1/2*) increased during the window of implantation suggesting a relevant role during this period. Data suggest that both ADIPOR1/ADIPOR2 are sensitive to energy status, as its expression was reduced in MA cows, reflecting the metabolic stress that these animals suffered during the transition period and are consistent with delayed reinitiation of ovarian cyclicity during the first month after calving (−30 to 30 DPP; Meikle et al., 2013).

Overall, the greater endometrial gene expression (IGF family and progesterone and adiponectin receptors) on day 7 of the oestrous cycle at the end of the voluntary waiting period found in TMR/LA when compared to MA/LA cows suggests a better physiological status of the endometrium in the former ones. This finding may improve embryo development and elongation, which in turn could impact on consequent delivery of IFNt and blockade of the luteolytic mechanism (e.g. embryo signalling, maternal recognition of pregnancy and lower embryo losses; Spencer, Forde, & Lonergan, 2015). Future research on the relevance of energy balance during the early post-partum period on uterine gene expression and function at the end of the voluntary waiting period may explain the lower fertility pregnancy rates found in lactating dairy cows.

## 5 | CONCLUSION

This study provides solid evidence of the importance of nutritional management during early lactation on uterine environment at the end of the voluntary waiting period. The greater expression of genes

related to embryo growth and uterine function (IGF system and progesterone and adiponectin receptors) in cows fed with diets allowing increased energy intake suggests a favourable environment for embryonic growth, which may explain the improved reproductive performance in cows in good energy balance.

## ACKNOWLEDGEMENTS

The authors thank undergraduate student for assistance with animal management and sampling, and the staff of the Experimental Station Dr. Mario A, Cassinoni for animal care and assistance throughout the work. The research that gives rise to the results presented in this publication was funded by the Agencia Nacional de Investigación e Innovación under the code ANII #POS_2011_1_3577 for the graduate scholarship awarded to A. Astessiano.

## CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

## AUTHOR CONTRIBUTIONS

All the co-authors collaborated in the generation of the model, in the organization of the field work and in the interpretation of the results. In particular, Mariana Carriquiry and Pablo Chiibroste collaborated in the final discussion of the data, and Ana Meikle collaborated from the experimental design to the interpretation and discussion of the results and in the final writing of the article.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Astessiano AL, Carriquiry M, Mattiauda DA, Adrien ML, Chilibroste P, Meikle A. Endometrial gene expression in primiparous dairy cows at the end of the voluntary waiting period is affected by nutrition: Total mixed ration vs increasing levels of herbage allowance. *Reprod Dom Anim*. 2017;00:1–8. https://doi.org/10.1111/rda.12981