RONAN LOPES ALBINO

MAMMARY GLAND DEVELOPMENT OF PREPUBERTAL CROSSBRED (HOLSTEIN X GYR) HEIFERS UNDER DIFFERENT GROWTH RATES

Thesis submitted to the Universidade Federal de Viçosa as partial fulfillment of the requirements of the Graduate Program in Animal Science to obtain the degree of Doctor Scientiae.

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Marta Fonseca Martins

Simone Eliza Facioni Guimarães

Márcio de Souza Duarte
(Co-adviser)

Kristy Marie Daniels

Marcos Inácio Marcondes
(Adviser)

DEDICATÓRIA

Dedico à minha família, à minha eterna companheira, Leonor e, à paixão da minha vida, Estela.

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BIOGRAFIA

Ronan Lopes Albino, filho de Rildo de Lima Albino e Marta Imaculada Lopes Albino, nasceu em Rio Pomba / MG - Brasil em 20 de janeiro de 1988.

Começou a graduação em Zootecnia na Universidade Federal de Viçosa (UFV), em 2007, e obteve o título de bacharel em julho de 2011. Em agosto de 2011, iniciou o programa de Mestrado em Nutrição e Produção de Ruminantes com foco em bovinos leiteiros da UFV. Entre Janeiro e Março de 2011 foi para Kentucky-USA realizar um estudo complementar ao seu trabalho de mestrado. Em julho de 2013, obteve o título de Mestre em Zootecnia.

Em agosto de 2013, iniciou o programa de doutoramento em Nutrição e Produção de Ruminantes com foco em bovinos leiteiros pela mesma universidade. Em 28 de julho de 2016, apresentou sua tese à comissão avaliadora, onde obteve o título de Doutor em Zootecnia.

EPÍGRAFE

"Não parar, não precipitar e não retroceder"

Olavo de Carvalho

SUMÁRIO

RESUMO

ALBINO, Ronan Lopes, D.Sc. Universidade Federal de Viçosa, Julho 2016. **Desenvolvimento da glândula mamária de novilhas mestiças (Holandês x Gir) prépúberes sob diferentes taxas de crescimento.** Orientador: Marcos Inácio Marcondes. Co-orientadores: Márcio de Souza Duarte, Sebastião de Campos Valadares Filho.

Este trabalho foi desenvolvido com o objetivo de estudar o efeito dos níveis de desempenho durante a recria de animais cruzados Holandês: Gir (H:G) sobre o desenvolvimento da glândula mamária; validar a técnica de ultrassom como ferramenta de monitoramento instantâneo e não-invasivo dos tecidos que compões o parênquima mamário; descrever o procedimento cirúrgico indicado para obtenção de amostras do tecido parenquimal da glândula mamária de novilhas em crescimento. O trabalho foi conduzido no Departamento de Zootecnia da Universidade Federal de Viçosa, Viçosa-MG. Dezoito novilhas mestiças (H: G) com peso inicial médio (102,2 ± 3,4kg) e idade entre 3 e 4 meses foram avaliadas ao longo de 84 dias. As novilhas foram, igualmente, distribuídas (n = 6) entre os tratamentos alto ganho (AG; 1,0 kg/dia), baixo ganho (BG; 0,5 kg/dia) e mantença (MA). A diferença de ganho de peso entre os tratamentos foi alcançada através da quantidade de alimento ofertada de uma mesma dieta a base de silagem de milho, farelo de soja, farelo de soja By pass e minerais com relação volumoso: concentrado de 60:40. O escore de condição corporal (ECC), a amostragem do volumoso e das sobras foram realizadas semanalmente. O peso das novilhas e as avaliação ultrassonográficas da glândula mamária foram realizadas em intervalos de 14 dias. As medidas corporais, as coletas de sangue e o ultrassom na carcaça foram realizadas em intervalos de 28 dias. Paralelamente, foi realizado uma biópsia na glândula mamária de 3 novilhas de cada tratamento no início e meio do experimento para se obter amostras representativas do tecido parenquimal. Ao final do experimento todas as novilhas foram abatidas e em sequência análises bioquímicas, histológicas e de expressão gênica foram realizadas na glândula mamária de cada animal. O consumo de matéria seca e demais nutrientes foi maior para os animais do AG em relação ao BG e este, por sua vez, maior do que MA, confirmando assim a efetividade dos tratamentos propostos. O ganho de peso apresentou comportamento semelhante ao consumo, sendo 0,90 kg/dia para AG, 0,50 kg/dia para BG e 0,10 kg/dia para MA. Novilhas do tratamento AG apresentaram maior ECC, espessura de gordura subcutânea (EGS) e área de olho de lombo (AOL) em avaliações ultrassonográficas da carcaça quando comparado aos demais tratamentos. Nas avaliações ultrassonográficas da glândula mamária houve maior concentração de pixels no parênquima mamário do AG em relação ao BG e MA, os quais não diferiram entre si. Novilhas do

tratamento AG apresentaram maior peso de gordura extra-parenquimal e maior concentração de extrato etéreo no parênquima em relação ao tratamento BG e MA. Estes resultados indicam, conjuntamente, uma maior concentração de tecido adiposo no parênquima de animais sob elevado ganho de peso. Com relação às avalições histológicas foi observado maior porcentagem de área ocupada por tecido epitelial no parênquima mamário das novilhas do tratamento BG em relação ao AG e MA. Não foi observado diferença na expressão gênica do IGF-1 entre os tratamentos AG e BG, porém houve maior expressão do TGFR βI no tratamento AG. Dessa forma, conclui-se que novilhas alimentadas para elevado ganho de peso, mesmo com adequado nível de proteína na dieta, acumularam mais gordura na glândula mamária e apresentaram maior expressão de genes que reduzem o crescimento epitelial. No segundo trabalho foi realizado uma análise de regressão da concentração de pixels da região parenquimal da glândula mamária (efeito fixo) em função das medidas de porcentagem de extrato etéreo no parênquima (PAR), porcentagem de proteína no PAR, área ocupada pelo tecido epitelial no PAR, número de adipócitos no PAR, peso da gordura extra-parenquimal e peso do parênquima (efeito aleatório) para estudar a relação entre as variáveis. Em seguida foi realizado uma análise de sensitividade para determinar a acurácia e precisão das equações encontradas. Dentre as variáveis testadas apenas peso de PAR não foi correlacionado com os valores de pixels na região parenquimal. Para as variáveis com correlação significativa foi observado alta acurácia e de média a alta precisão. Imagens de ultrassom permitiram identificar diferentes padrões de crescimento do tecido parenquimal mamário influenciadas pelo nível de desempenho das novilhas. Por fim, foi feito uma descrição dos procedimentos empregados para obtenção de amostras do tecido parenquimal mamário de novilhas em crescimento. Após a amostragem do tecido PAR procedeu-se a extração do RNA e DNA de cada amostra. A integridade das amostras foi comprovada pela relação de absorção entre oRNA (260 nm) e de proteínas (280 nm) acima de 1,80, indicado a qualidade do RNA obtido.

ABSTRACT

ALBINO, Ronan Lopes, D.Sc. Universidade Federal de Viçosa, July 2016. **Mammary gland development of prepubertal crossbred (Holstein x Gyr) heifers under different growth rates.** Adviser: Marcos Inácio Marcondes. Co-advisers: Márcio de Souza Duarte, Sebastião de Campos Valadares Filho.

This work was designed to study the effect of performance levels during rearing on the mammary gland development of Holstein: Gyr (H: G) crossbred heifers; validate the ultrasound technique as a feasible tool to monitor instantaneously and non-invasive the parenchymal composition of mammary gland; describe surgical procedure for obtaining the parenchymal tissue samples from mammary glands of growing heifers.The work was performed in the Animal Science Department of Universidade Federal de Viçosa, Viçosa-MG. Eighteen crossbred heifers (H:G) with initial weight (102.2 ± 3,4kg) and aged between 3 and 4 months were evaluated over 84 days. Heifers were equally distributed (n = 6) between the high-gain treatments (HG, 1.0 kg / day), low gain (LG, 0.5 kg / day) and maintenance (MA).The difference in weight gain between treatments was achieved by the amount of feed offered using a single diet based on corn silage, soybean meal, soybean meal and minerals By Pass with roughage: concentrate ratio of 60:40.The body condition score (BCS), sampling of silage and leftovers were taken weekly. The heifer's weight and ultrasonographic evaluation of the mammary gland were performed in 14 days intervals. The biometric measurements, blood samples and ultrasound on carcass were performed in 28 days intervals.In parallel, a biopsy was performed in mammary glands of heifers (3/treatment) at the beginning and middle of the experiment to obtain representative samples from the parenchymal tissue. At the end of the trial, all heifers were slaughtered and following biochemical, histological and gene expression analysis were performed in the mammary gland of each animal.The dry matter intake and other nutrients was higher for HG heifers when compared with LG and this, in turn, higher than MA, confirming the effectiveness of proposed treatments. Weight gain behaved similarly to intake, being highest gain for HG heifers (0.90 kg/d), 0.50 kg/d for BG and 0.10 kg/day MA.HG heifers showed higher BCS, subcutaneous fat thickness (SFT) and ribeye area (REA) in carcass evaluation when compared to other treatments. In ultrasonographic evaluation of the mammary gland there was a higher pixels concentration in HG mammary parenchyma than BG and MA, which did not differ from each other. HG heifers showed higher extra-parenchymal fat weight and higher concentration of ether extract in the parenchyma than BG and MA heifers. These results indicate, together, a higher concentration of fat in the parenchyma of animals under high weight gain. Regarding to

histological evaluations in mammary parenchyma was observed higher percentage of area occupied by epithelial tissue in LG treatment than HG and MA. There was no difference in gene expression of IGF-1 between HG and LG treatments, but there was a greater expression of TGFR βI in HG.Thus, we concluded that heifers fed to show high weight gain, even with adequate level of protein in diet, accumulated more fat in mammary gland and had greater expression of genes that reduce epithelial growth.In the second study was conducted a regression analysis of pixels concentration in mammary parenchyma (fixed effect) in function of ether extract percentage in parenchyma (PAR), protein percentage in PAR, area occupied by epithelial tissue in PAR, number of adipocytes in the PAR, weight of extra-parenchymal fat and weight of parenchyma (random effect) to study the relationship between variables.At sequence, a sensitivity analysis was performed to determine the accuracy and precision of the equations found. Among the variables tested only PAR weight was not correlated with the pixel values in the parenchymal region. Between variables with significant correlation was observed high accuracy and from middle to high precision. Ultrasound images allowed identifying different growth patterns of mammary parenchymal tissue influenced by the level of performance of the heifers.Finally, it was described the procedures used to obtain samples of mammary parenchymal tissue in growing heifers. After sampling was carried out the RNA and DNA extraction from each PAR.Relation between RNA absorption (260 nm) and protein absorption (280 nm) analyzed the integrity of samples. The ratio greater than 1.80 indicated the quality of the RNA obtained.

GENERAL INTRODUCTION

Heifer rearing is part of dairy herd turnover. The length between birth and calvingit is a crucial point of profitability of dairy herd, once that time will determine the selection pressure and growth rateof the herd (Mohd Nor et al., 2014). The shortening of rearing period would lead to cost reduction (Hoffman et al., 1996). This can be achieved by faster heifer growth, earlier onset of sexual maturity, breeding and calving. However, accelerated heifer growth can be associated with excessive fat deposition in the mammary gland and then impair milk yield after the first calving (Radcliff et al. 2000). An explanation to reduced milk is associated with fat accumulation due to an increased proportion of fibroblasts and adipocytes rather than epithelium(Turker, 1987).

At birth, calves have rudimentary mammary system, which is composed almost entirely of fat pad (Meyer et al., 2006). Over the heifers' rearing phase, the mammary gland shows two types of growth, isometric and allometric. During isometric phase the mammary gland grows 1,5 Fold faster than body and in the allometric phase the gland grows from 3 to 4 Fold faster than body. The isometric phase goes from birth to 2 months old and the allometric begins with 2 months and goes until onset of the puberty (Sinha and Tucker, 1969). The calf epithelial tissue develops progressively becoming entangled between to the fat padthrough the terminal duct lobular units, which in the future will become in alveoli. Thus, when evaluatingPARsamples of weaned heifers there is always the presence of fat pad intermingled with epithelial and connective tissues.

The mass of the mammary fat pad is probably directly related to the high weight gain during the pre-pubertal period. This in turn is directly related to the amount of energy consumed by the animal. However, some authors have suggested that the main influence on the accumulation of fat in the mammary gland is not specifically energy intake, but rather the proportion between the energy and the protein consumed by the animal (Capuco et al. 1995; Dobos et al. 2000).

Hoovey et al. (1999) compared the growth and function of the fat pads in different mammalian species, including cattle. These authors highlight the importance of the fat pad for the perfect development of the epithelium, since it is involved in the synthesis of growth factors, support growth factor receptors, as well as being a direct source of lipids and nutrients for growth mammary. Hoovey et al. (1999) showed that the number of epithelial cells within the mammary gland are coordinated by the amount of fat in fat pad. Considering

that milk production is proportional to the number of epithelial cells (Akers, 1990), the volume of fat pad could be a tool to determine the future productive potential of the heifer.

Despite of the fat pad has a crucialrole at growth and branching of epithelial cells is still unclear how much fat pad that would be necessary for the full mammary gland development and which would become a limiting factor for PAR growth.

Some researchers suggest that there is no deleterious effect of weight gain on mammary development when diets with proper balance between energy and protein are offerto heifers (Capuco et al, 1995; Dobos et al, 2000; Whitlock et al, 2002). On the other hand, there are studies that found a reduction in milk production when heifers are rearing in high performance nutrition plans (Lammers et al., 1999).

This contradiction of literature results can be resultof rearing time in which heifers are evaluated (Meyer et al 2006), by the experiments duration and the different objectives set out in the design of experiments. Zanto and Heinrichs (2005) conducted a meta-analysis of 15 studies published between 1990 and 2005 in order to verify the influence of the GMD on milk production at first lactation. The authors concluded that superior performance 800 g/day reduce milk production at first lactation.

Monitor continuously the growth of mammary gland could be helpful to understandthe correlation between the composition of the gland and the eating plan in order to predict their impact on milk production at first lactation. Several authors have tried to establish such correlations, but all work presented problems in assessing the way. The most of works that focus on monitoring the gland used slaughter and did not follow the production of heifers after delivery. In contrast, the work that accompanied the production of heifers not used methods that could evaluate accurately the mammary glands of heifers (Lammers et al., 2000).

Therefore, we proposed evaluate the effects of performance in heifers fed with an adequate balance between energy and protein on diet under mammary gland. In addition, we seek to test the use of ultrasound as a tool to estimate instantaneously and Noninvasive the performance effect on breast development

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CHAPTER 1

PERFORMANCE STRATEGIES FOR CROSSBREED HEIFERS

Interpretive summary

The heifers' weight gain through rearing phase isdefinedby energy intake. Furthermore, energy intake will influence the age at first calving and the herd turnover, in turn, impact the system efficiency. The milk production after heifers' calving can be handled in the rearing phase through of adjust between energy: protein consumed by heifer, avoiding offer an excessive energy on diet that will stimulate the fat accumulation in mammary gland and impair the yield potential. The influence of excess energy intake on mammary gland development have been extensively studied in Holstein heifers, but almost no studied was performed with crossbred.

Performance Strategies Affect Mammary Gland Development in Holstein: Gyr Crossbreed Heifers. By Albino et al.

R. L. Albino*, A. L. Sguizzato*, K. M. Daniels[†], M. S. Duarte*, M.M. Lopes[*], S. E. F. Guimarães, M. M. D. C. A. Weller*, M. I. Marcondes*[1]

*****Universidade Federal de Viçosa,Viçosa, MG, Brazil, 36570-900

[†]Dairy Science Department, Virginia Polytechnic Institute and State University, Blacksburg 24061

[1]Corresponding author: marcosinaciomarcondes@gmail.com

ABSTRACT

Several studies have verified whether high performance during the prepubertal rearing phase affects mammary gland development and therefore milk production at first lactation.However, most of these were unable to isolate the effect to diet from performance, since differences in performance were caused by changes in the composition of diets. Thus, our hypothesis is that heifers fed with proper dietary energy protein ratio, if subjected to high weight gain during the prepubertal period, show no reduction in mammary parenchyma development. To test our hypothesis, development of the mammary gland from crossbred heifers under different performance strategies was monitored by ultrasound images, gene expression and histology. The experiment was carried out in a completely randomized design, with 6 heifers per the treatments randomly assigned to high gain (**HG** 1 kg / day), low gain (**LG**, 0.5 kg / day) and maintenance (**MA**) treatments. Over 84 days feed intake, BCS, weight gain and body measurements growth were monitored. Feed samples, blood and mammary parenchyma (**PAR**) tissue were obtained throughout the experiment. Mammary gland and carcass ultrasound images were taken at intervals of 14 and 28 days. At the end of the experiment all heifers were slaughtered and samples of mammary PAR were obtained and either stored in RNA-holder and buffered formalin solution (10% v/v) for further IGFR1, IGF1 and *TGFRβ1* gene expression and histological analyses. Lastly, PAR tissue was subjected to chemical analysis. Heifers HG treatment showed significantly higher BCS compared to LG (P=0.001) and MA (P=0.001). Heifers HG showed higher pixels value in the PAR area compared to LG (P=0.001) and MA (P=0.001). PAR weight (P=0.076) and protein percentage (P=0.092) were higher in LGthan HG heifers. On the other hand, the fat weight (P=0.001) and ether extract percentage (P=0.056) were greater in HG than LG heifers. The area occupied by epithelial tissue in the mammary gland PAR was higher in LG than HG (P=0.027) and MA (P=0.044) heifers.No difference were observed for parenchymal IGF-1 gene expression (P=0.200) between HG and LG heifers. However, the gene expression of TGF-βR1on PAR of HG was higher than in LG (P=0.006) and MA (P=0.004) heifers. These results indicate that heifers subjected to high weight gain during rearing period even under adequate dietary energy: protein ratio, would experience a impairment of mammary development.

Key words:heifer, mammary gland, diet

INTRODUCTION

Fat accumulation in mammary glands due to excessive energy intake is well documented in Holsteins (Meyer et al., 2006; Rinker Davis et al., 2008) and energy intake is a major regulator of weight gain (Owens et al., 1995). A meta-analysis of 15 studies published between 1990 and 2005 concluded that body weight gains in excess of 800 g/day have potential to reduce milk production (Zanto and Heinrichs, 2005). In Brazil, the majority of dairy cattle are Holstein x Gyr (H:G). It is unknown whether excessive energy intake negatively affects mammary development.

Mammary development in cattle is typically quantified after slaughter by measurement of tissue mass, composition, and relative abundance of genes associated with tissue growth (Brown et al., 2005; Meyer et al., 2006; Daniels et al., 2009). These approaches have obvious drawbacks, the main one being inability to measure eventual milk production. Recent studies (Esselburn et al., 2015), including our own work (Albino et al. 2016) have explored non-invasive means to measure mammary development through ultrasonography (**US**).

In addition to affecting mammary growth and composition, heifer nutrition programs for Holsteins can also impact concentrations of circulating blood metabolites such as insulin-like growth factor-I (**IGF-I**) (Whitlock et al., 2002; Radcliff et al., 2004; Daniels et al., 2009). As with mammary development, the impact of dietary energy on concentrations of blood IGF-I is not well characterized in H:G. Knowledge of how blood IGF-I is affected by nutrition in H:G is valuable.

Similar to previous work conducted with Holsteins, our hypothesis was that mammary development, blood metabolites, and stature of H:G heifers can be impacted by dietary energy supply. Therefore, our aim was to evaluate the effect of different average daily gains achieved by feeding different amounts of a common diet during the growing period on: 1) development of mammary parenchyma (**PAR**) and mammary fat pad (**MFP**); blood IGF-I; 3) and biometric measurements.

MATERIALS AND METHODS

Experimental Design

The 84 d experiment was conducted in the Animal Science Department of theUniversidade Federal de Viçosa, Viçosa, MG, Brazil.The institutional ethics committee approved the experiment (protocol number 20/2015). Before treatment assignments and the experimental period, all heifers had ad libitum access to an adaptation diet consisting of corn silage, corn-based concentrate, soybean meal and minerals with roughage/concentrate ratio of 60/40. Additionally, all heifers were treated for endo- and ecto-parasites with 1 ml /50 kg BW of Dectomax (Pfizer USA, Doramectin 1%).

At the end of the adaptation period, H:G heifers (n = 18) weighed 102.2 ± 3.4 kg and were 3 to 4 mo of age. At this time, heifers were randomly assigned to one of three average daily gain programsusing a completely randomized design.

Treatments were: high gain (**HG**, n = 6) where heifers were fed to gain 1 kg/day; low gain (**LG**, n=6), where heifers were fedto gain of 0.5 kg/day; and maintenance (**MA**, n = 6), where heifers were fed to gain a minimal amount of weight per day. The Dairy NRC (2001) was used to formulate a single diet that meet CP, rumen degradable protein (RDP), rumen undegradable protein (RUP), TDN, and macro- and micro-minerals of HG heifers (Table 1). Heifers on LG and MA were fed lower daily amounts of the single diet. MA heifers were fed sufficient feed to meet maintenance needs and allow for a marginal ADG of 100 g/d.

HG heifers were fed for 5% refusals (as-fed). Daily intake of LG and MA heifers was controlled such that no refusals remained; this facilitated desired daily body weight gains. All heifers were weighed every 14 d and diet amount adjusted accordingly.

Biometric Measures and Body Condition Scores

Heifer body condition scores(**BCS**) were evaluated weekly using a 5 point scale by three trained technicians; the average score for each heifer is reported for each timepoint (Edmonson et al., 1989). Other biometric measurements including, withers height (**WH**), body length (**BL**) and heart girth (**HG**) were performed in 28-day intervals.

Feed Analysis

Corn silage samples werecollected weekly and partially dried in a forced air oven at 55°C for 72 h to determine dry matter (**DM**) content; weekly DM measurements were used to

ensure ration the roughage to concentrate ratio was . Thus, we performed the weekly correction of roughage DM content in order to adjust roughage/concentrate ratio on diet.

Samples of soybean meal, corn and By Pass soybean meal that composed concentrate were collected twice along the experiment during mixing process in the feed mill. Samples of diet remains from HG treatment were partially dried in an oven with forced air ventilation at 55°C for 72h. Fecal samples were obtained by total collection during three consecutive days in two digestibility tests performed between days 25 to 27 and 53 to 55 after experiment beginning. Fecal samples were also partially dehydrated in an oven with forced air ventilation at 55°C for 72h.Urine samples were taken at same two digestibility trials using three sampling time per day (spot) 6:00, 12:00 and 18:00 o'clock, during 3 days (Valadares et al., 1997).

All feed samples, leftovers and feces were ground in knives-type mill using a 1 mm sieve and subsequently submitted to analysis of dry matter (DM) (AOAC, 1990; number 920.87), ash (AOAC, 2005; number 942.05), crude protein (CP) (AOAC, 2005; number 990.13), insoluble neutral detergent fiber (NDF) (Mertens, 2002) with correction for ash and protein, and ether extract (EE) (AOAC 2005; number 2003.05).

Non-fibrous carbohydrates (NFC) content were obtained by equation (Detmann & Valadares Filho, 2010) considering the correction for ash and NDF protein (NDFcp), and the correction for urea CP (CPu) and the amount of dietary urea (u).

$$NFC = 100 - (NDFcp + (CP - CPu + u) + EE + MM)$$

Blood Metabolites Analyses

Blood samples were collected on days 0, 28, 56, 70 and 84 by puncturing jugular vein prior morning feeding. Blood was collected in 2 tubes of 10 mL without anticoagulant in order to separate serum from blood. After sampling, samples were centrifuged at 1,304 x g for 15 minutes and serum samples (1 mL)were transferred into microtubes and separately frozen at -20 °C. The IGF1 concentrations were determined with a chemiluminescence assay (IMMULITE 2000 IGF1 Siemens Healthcare Diagnostics Products Ltd.,Gwynedd, UK). Serum insulin was determined using an immunochemiluminescence assay kit supplied by Abbott (Abbott Japan Company Ltd., Tokyo, Japan). Levels of Estrogen (E2) and progesterone (P4) in blood serum were determined by immunochemiluminescence method using Access Systems (Beckman Coulter, Inc., 250 S. Kraemer Blvd., Brea, CA 92821 USA). From day 56 to day 84, blood samples were collected from all heifers in 14 days intervals for

P4 analysis. Successive results of P4 higher than 1 ng/ml were interpreted as heifer presenting functional corpus luteum, and therefore the pubertal stage started (Daniels et al., 2006). Growth hormone (**GH**) and insulin like growth factor binding protein 3 (**IGFBP3**) were not detected in the immunochemiluminescence assay.

Mammary GlandUltrasound Evaluation

Mammary gland development was evaluated in 14 days intervals using ultrasound (B-mode) equipped with micro-convex transducer operating at a frequency of 6.5 MHz (DP2200, Mindray, China) as described in Albino et al. (2016, under review, JDS 16-11668) .

Two images were taken from each teat, from which we selected the image with better resolution. Finally, four selected images of each heifer per day of evaluation were analyzed on ImageJ® software (National Institutes of Health, Bethesda, Maryland, USA). Within each image we collected six random squares of 0.4 cm wide by 0.4 cm high, being three located at the PAR area and three immediately above, at MFP area. Within each square, pixel concentration was used to calculate pixel average separately for each squares from ductal and sub ductal region. Pixels scale was calibrated through a non-inclined trace which considered 1 cm equal to 50 pixels.

Mammary Tissue Sampling

On days 0 and 42 after experiment beginning, a biopsy was performed for parenchymal tissue sampling from mammary glands of heifers. Three heifers from each treatment were randomly chosen. Before the procedure, heifers were maintained in solid fasting for 12 hours. Each heifer was individually contained and applied 0.5 ml/100 kg intramuscular dose of xylazine-based general anesthetic. Then, heifer was lying in lateral position, restrained with ropes and mammary gland sanitized with 70% alcohol. Subsequently, the outer side of left mammary hindquarter was shaved and 10 ml of local lidocaine-based anesthetic applied. After anesthetic effect, a 5 cm incision, approximately 5 cm above of teat base, was made with a scalpel on the gland surface; approximately 50 mg PAR was collected as far as it was identified. Finally, samples were immersed in Falcon tube containing 5 mL RNA holder solution (RNAholder, Bio Agency Biotechnology LTDA, São Paulo), kept at 4°C for 24 hours and then stored at -80°C until RNA extraction. Biopsy of day 42 followed the same pattern, but in the right hindquarter.

At 84 days, all heifers were slaughtered. After slaughter, mammary gland was completely removed and weighed. Then, skin and teats were removed and weighed separately. Subsequently, gland was divided along medial ligament and each half weighted. PAR weight was recorded after separation of MFP and lymph nodes. Fragments of PAR tissue were removed from hindquarters (left or right) of each mammary gland and stored as described above for RNA extraction. After sampling RNA fragments, samples of PAR were taken from PAR remaining hindquarters and immediately fixed in formalin solution (10% v/v) at pH = 7.4 for 24 hours to later histology analysis. Then, samples were stored in ethanol 70% (v/v) solution up to their inclusion in paraffin.No visual fibrosis, due to biopsies performed before, was identified at PAR tissue sampling after the slaughter. We care to identify the PAR region to carry out the sampling for RNA and histology analysis.

Finally, PAR tissue remaining was submitted to analysis of DM (AOAC, 1990; number 920.87), CP (AOAC, 2005; number 990.13) and EE (AOAC, 2005; number 2003.05). Immediately after sampling, tissues were separately stored and frozen at -20°C until analysis. For chemical analysis, we first unfrozen samples for 1 hour at room temperature. Each sample was grounded under liquid nitrogen and lyophilized.

Chemical and Ultrasonographic Evaluation of Body Composition

In 28 days-intervals heifers were measured ultrasonically on the right side body by an expert technician using an Aloka (model: SSD 500V, Aloka Co., Ltda., Tokio, Japan) with a 18 cm, 3.5 MHz linear probe. The subcutaneous fat thickness (**SFT,** in mm) and ribeye area (**REA,** in cm^2)on the longissimus dorsi muscle were measure at the rump. Images were analyzed into BioSoft Toolbox II for Beef (Biotronics Inc., Ames, Iowa, USA) (Realini et al., 2001).

After slaughter, carcass was split into two identical longitudinal halves. After a 24-h chill, the right half of the carcass was reduced to smaller pieces and ground through a mixer/grinder. After fine grinding and individual homogenization, two composited samples (100 g each) were collected and freeze-dried (80 h) for dry matter determination.Then these samples were ground again with liquid nitrogen and analyzed for fat (by loss in weight of the dry sample upon extraction with diethyl ether in Soxhlet extraction apparatuses for 10 h; AOAC, 1990), protein (nitrogen analysis via macro Kjeldahl using 1.5 g of sample; AOAC, 1990), and ash (complete combustion in a muffle furnace at 600°C for 16 h; AOAC, 1990)

Gene Expression and Histological Analysis

Gene expression and mammary histology analyses were carried out in partnership with Weller et al. (2016). All procedures involving RNA extraction, cDNA synthesis, and quantitative real-time reverse transcription PCR (qRT-PCR) are described by Weller et al. (2016). cDNA prepared from PAR was used in this analysis. Target genes were: IGF1, IGF1Rand TGF-βR1.Internal control genes wereHPRT1 and RSP15A(Weller et al., 2016). Primer pairs for all genes are listed in Table 2. Formalin-fixed, paraffin embedded, hematoxylin and eosin stained PAR was used here; adipocyte number in PAR and area occupied by epithelium were tabulated as reported in Weller et al. (2016).

Statistical Analysis

Hormonal and carcass ultrasound data obtained in 28-day intervals, pixels obtained in 14-days intervals, and weekly average of BCS were analyzed using a completely randomized design as repeated measurements in time, according to the following model. Weekly average of BCS were generated from the average score of three experienced technicians and tested for normality ($P>0.05$; Davis, 1989) (data not shown). Pixel values did not follow a normal distribution, therefore, we standardized the values using log10 transformation ($P<0.05$; Davis, 1989).

$Y_{ike} = \mu + T_i + \delta_{ijk} + P_e + (TxP)_{ie} + \varepsilon_{ike}$

μ = general mean

T_i = fixed effect of treatment

δ_{ijk} = random error with average 0 e variance σ^2 δ, between animals within treatment is the covariance between repeated measurements of animals

P_e = fixed effect of period,

$(TxP)_{ie}$ = fixed effect of treatment i and period e interaction

ε_{ike} = random error with average 0 e variance τ^2, between measurements and animals.

Serum hormone concentrations and qRT-PCR was analyzed according Weller et al. (2016). Means were tested using the PROCMIXED option of SAS and were considered significant when $P \leq 0.05$ and tendency when $0.05 \leq P \leq 0.10$. The IGF1, IGFR1 and TGF-βR1 gene expression were test in day 0 and was not observed difference(data not shown).

For variables related to chemical and physical evaluation of mammary gland were considered significant $P \leq 0.10$. This criterion was adopted due the difficulty of sample handling and physical separation of PAR from MFP.

Initial live weight was initially used as a model variable; as no significance was identified in any of the cases ($P>0.05$) it was excluded from the models.

RESULTS AND DISCUSSION

In this study, diet was formulated for animals with 130 kg of body weight, and average daily weight gain of 1.0 kg/day (NRC, 2001). However, average body weight observed between treatments was 122.2 ± 5.7 kg, being 143.1 ± 59.5 kg for HG, 125.3 ± 52.7 kg for LG and 102.6 ± 44.5 kg for MA. Table 3 presents the nutritional requirement based on planned and observed weight according to NRC (2001), and the results observed in this experiment to animals with ad libitum consumption.

Observed and predicted values of DM and CP were similar, showing that young crossbred heifers fed in total confinement have potential intake similar to Holstein heifers (NRC, 2001). Intake of dry matter, organic matter (OM), CP, NDF and NFC were different ($P<0.05$) between treatments (Table 4). These results are consistent with the proposed experimental design, in terms that difference in nutrient intake was part of expected difference between treatments. Heifers from HG treatment consumed an average of 2.65% of DM in relation to body weight (BW). Heifers from LG and MA treatments consumed 2.04 and 1.44% of DM in relation to BW, respectively. Intake of heifers from LG and MA treatment was controlled according to metabolisable energy requirement that would allow an average daily gain of 0.5 kg and about 0.1 kg/day (Table 4), respectively.

Heifers from HG treatment gained 100 g/day below the expected at the experiment beginning (1.0 kg/d). Regarding LG treatment, observed performance was as planned. Heifers from MA treatment gained 100 g/day of body weight, showing the expected effect on performance (Table 4).

Gains of BL, GH and WH were different between treatments ($P<0.05$). Heifers fed to higher weight gain need also increased bone structure growth, for weight gain is not obtained only by body fat accumulation (Gabler & Heinrichs, 2003; Casper et al., 1994). Moreover, despite heifers from HG have increased ADG, which could be interpreted as greater fat accumulation if only evaluating the extra weight, they also had higher body growth rate, which in turn, contributes to disperse the body fat accumulation (Table 4).

We clearly observed that heifers from HG had higher BCS ($P<0.05$) throughout the experiment than animals from LG and MA treatments (Figure 1A). BCS is a subjective and indirect method of measuring body fat accumulation, obtained by assigning a score, which ranges from 1, for extremely lean animals, up to 5, for extremely fatty animals. BCS is a well-used tool by nutritionists and farmers to monitor the nutritional status of dairy cows, which should be between 3 and 3.5 (Garnsworthy, 2007). However, the use of BCS for growing heifers is little documented in literature, as well as the optimal level of BCS for heifers at different stages of growth. Nevertheless, there is a practical consensus that BCS of growing heifers should be less than ideal BCS of cows; values between 2.5 and 3 are used as a reference in some practical notes published for growing heifers (Kellog, University of Arkansas, undated file extension).

The lowest BCS for heifers can be justified by the higher deposition of protein tissue compared to fatty tissue observed in animals at initial growth stage (Owens et al, 1995). Animals from LG and MA were closer to optimal values of BCS. On the other hand, HG animals presented values higher than desirable for heifers during most of the experiment, indicating excess of body fat accumulation and a possible accumulation of fat in mammary gland (Figure 2).

Even though BCS being a subjective method to estimate body fat accumulation, the same results were indicated by ultrasound in carcass, that measures objectively the accumulation of fat and lean in the tissue (Figure A and B). Subcutaneous fat thickness (SFT) of HG was higher ($P<0.001$) than LG, which in turn did not differ ($P=0.083$) from MA. Effect of treatment by period interaction (T*P) is explained by SFT gain of HG and LG heifers over time, followed by the maintenance of SFT gain in MA treatment, which reinforces the effect of experimental design on the results (Figure 1A). Regarding rib eye area (REA), HG heifers showed higher values ($P=0.004$) than those observed in LG heifers, which did not differ ($P=0.574$) from MA treatment (Figure 1B).

In order to associate ADG (Table 4) and BCS (Figure 2) with mammary fat accumulation, we evaluated the concentration of pixels in PAR and MFP regions of heifer's mammary gland (Figure 2). Variation of images echogenicity indirectly indicates the proportion of specific tissues in the structure. Images generated with high pixels values are associated with increased accumulation of fat in mammary gland, due to greater fat reflection of ultrasound waves (Albino et al., 2015, Albino et al., 2016; Esselburn et al., 2015).

Pixel values in PAR region of HG heifers were higher (P<0.001) than pixel values observed in LG and MA images. No difference was observed (P>0.05) between LG and MA heifers. In MFP area, pixel values between HG and LG were not different (P>0.05); HG (P<0.001) and LG (P=0.001) showed higher values than in images of MA heifers (Figure 2).

Albino et al. (2015) evaluated the effect of metabolisable protein:metabolisable energy (MP:ME) ratio on dietunder fat accumulation in mammary gland of pre-pubertal Holstein heifers by monitoring pixel concentration in mammary gland images. Heifers fed less than 38g MP/Mcal of ME (MP:ME ratio) showed an increase of pixels concentration in ultrasound images. The ratio shown in the above experiment is equivalent to a diet with 15% of CP in DM approximately. Dietary CP content used in this experiment (Table 1) was higher than values indicated as minimum threshold to avoid mammary gland fat accumulation (Albino et al., 2015). Thus, we expected that higher dietary protein intake would inhibit fat accumulation in mammary gland or stimulate protein accumulation in mammary gland. However, different than expected, the higher BCS of HG treatment heifers (Figure 1A) was similar to higher fat accumulation in mammary gland(Figure 2). Therefore, the results presented in this study indicate that even with an adequate energy-protein balance, there is a greater fat deposition in both body and mammary, together with high weight gain.

To reinforce the correlated events, physical composition of mammary gland, chemical composition of gland PAR, and body composition in heifers obtained after slaughter were evaluated (Table 5). In physical evaluation of mammary gland there was weight difference between treatments (P<0.001). The weight of LG treatment PAR was higher than HG (P=0.076) and MA (P=0.009), which did not differ each other (P=0.341). For MFP weight, HG treatment was higher to LG (P=0.001), being these higher than MA (P<0.001; P=0.012, respectively).

No differences were observed on chemical evaluation of PAR for levels of ash (P=0.132) and DM (P=0.681). On the other hand, CP concentration of HG treatment was lower than LG (P=0.092) and LG was lower to MA (P=0.076). Regarding to the EE content, HG treatment was higher to LG (P=0.056) and MA (P=0.002) treatment, which did not differ each other (P=0.117; Table 5). The CP content on HG (P=0.011) and LG body (P=0.055) were lower to MA. Opposite, HG (P=0.011) and LG (P=0.012) were higher to MA treatment in EE content on body (Table 5). The mammary gland consist of two major types of tissues that serve different functions. As summarized by Esselburn et al. (2015), these are: 1) epithelial tissue and surrounding stromal elements, collectively called PAR, and 2) stromal tissue that lies adjacent to PAR and does not contain epithelial structures, and instead, mainly

consists of connective structures and it is called MFP. Once observed that HG heifers showed a higher amount of fat in mammary gland associated with a lower PAR weight, it can be inferred that a considerable part of energy consumed by HG has been deposited as fat in mammary gland that may have affected the epithelium growth. Performing the same comparison, but only in gland PAR tissue, it is observed, likewise, that higher EE content is associated with lower protein content in PAR of HG compared to LG treatment.

These results reinforce the rejection of our hypothesis and more clearly indicate that high performance of crossbred heifers during prepubertal period tend to accumulate fat in mammary gland. It is noteworthy that in this experiment, unlike previous studies (Whitlock et al., 2002, Meyer et al., 2006, Rinker Davis et al., 2008), performance effect was undoubtedly isolate from diet effect. Thus, regardless the protein-energy on diet, it might be suggested that fat accumulation in both body and mammary gland is observed inherent to animals' weight gain.

Inferences made so far are based on results in macro level, which indicate the feeding plan effect only on tissue composition of body and mammary gland. Therefore, it is necessary to detect whether fat levels in HG heifers gland adversely affect cell synthesis in the mammary parenchyma.

Thereby, first we sought to evaluate performance effects on mammary gland composition, through PAR histology. Less number of adipocytes($P=0.008$) was observed in PAR of LG heifers compared to MA. On the other hand, there was no difference ($P=0.235$) between HG and LG heifers (Figure 3D). During postnatal stage, pre-adipocytes develop in two forms, hyperplasia and hypertrophy (Dodson et al., 2015). The fact that MA animals presented greater number of adipocytes compared to LG heifers may be explained by the difference in adipocyte growth due to energy consumption. Adipocytes are the primary source of storage of excess energy consumed (Azain, 2003). Thus, heifers with higher energy intake have increased accumulation of triglycerides into their adipocytes, which in turn increases adipocyte size. Then, by counting adipocytes number within a certain fixed area, it is observed that HG heifers had larger adipocytes, resulting in less number of adipocytes per area. Animal nutritional status modified the proliferative, adipogenic and lipogenic capacity of vascular stroma cells on in vitro studies, demonstrating that pre-adipocyte proliferation was higher in cells of not supplemented than supplemented steers (Kadegowda et al., 2014). The fact that animals with higher energy intakeshown lower number of adipocytes than MA animals, but adipocytes with greater accumulation of lipids can be confirmed by the greater ether extractcontentobserved in PAR of HG compared to LG heifers (Table 5).

Regarding area occupied by epithelial cells, we observed higher epithelium proportion in PAR of LG heiferscompared to HG (P=0.027) and MA (P=0.044), which did not differ from each other (P=0.733; Figure 3E). Despite HG and MA heifers did not differ (P=0.08) in epithelial cells area, it is suggested that opposing factors have caused these differences to LG. To HG heifers, excess of hypertrophy may have reduced epithelial cells development. On the contrary, for MA animals this effect occurred due to hyperplasia, or large numbers of cells with less adipocyte.

In summary, histological analysis indicated a reduction of epithelial PAR area and an increase in adipocytes size of high performance heifers during growing period. In addition to composition study of microscopic structure of tissue PAR, we also aimed to correlate the performance effect with blood parameters and genes expression related to tissue growth.

Heifers from HG and LG treatments did not differ (P>0.05) on insulin level of blood serum, however, both were higher (P= 0.007 and P=0.029) than MA treatment (Table 6). Concentration of insulin and IGF-1 might be used as an indicator of animal nutritional status. Blood increased insulin in higher performance animals is related to density of energy consumed (Lacasse et al., 1994; Yelich et al., 1996). Thus, with this blood parameter, it is possible to physiologically confirm the effect of the proposed treatments in the experimental design.

Ovarian estrogen secretion has a direct effect on growth and branching of heifers mammary ducts. Ovariectomized heifers had restricted growth of mammary tissue, being, however, observed normal return of mammary growth under exogenous estrogen administration (Capuco & Akers, 2010). Differences between treatment performances were not sufficient to alter serum estradiol levels (P>0.05) (Table 6). However, it is important to note that much of serum estradiol results were below of minimum threshold detection (20 pg/mL, n=26/52). Thus, there was a significant reduction in number of samples available for comparison, which in turn reduced the statistical powerfor detecting differences.

On day 0, there was no difference (P<0.05) between serum levels of IGF-1, indirectly indicating similar nutritional status at the beginning of the experiment (Figure 4). On days 42 (P=0.008) and 84(P=0.008) IGF-1 level on serum of HG was higherthan LG heifers. Serum levels of IGF-1 of LG and MA heifers did not differ (P>0.05) across all evaluated days (Figure 4).

IGF-1 is mainly produced in liver by stimulus release from GH. However, IGF-1 can also be produced in tissues where it operates. According to Berry et al. (2001), there is significant production of IGF-1 in mammary gland of growing heifers, being this responsible

by the growth hormone mitogenic effect in the mammary gland(Berry et al., 2001; Berry, et al., 2003).Although higher levels of IGF-1 in bloodstream may indicate increased mitogenic activity in body tissues, this should be carefully evaluated in terms that IGF-1 effect is firstly coordinated by their binding factors, as well as the place where it operates (Purup et al., 2000). IGFBP3 is main biding factor associated with IGF-1, and it has the roleof enhancing IGF-1action on serum and inhibiting it effect on mammary gland (Purup et al., 2000). Therefore, we sought to evaluate gene expression in PAR tissue to confirm the effect of IGF-1 and other genes involved with mammary parenchyma growth (Figure 5).

We did not observed a period*treatment interaction effect in mRNA expression IGF-1 (Figure 5A), thus treatment effect was presented separately from period effect. Low gain heifers showed increased mRNA expression of IGF-1 (P=0.036) compared to MA. For HG, mRNA expression of IGF-1 was intermediate between LG and MA. PAR is a set of tissue consisting of epithelial, connective and adipose tissue (Weber et al., 2000).The IGF-1 receptors are identified in a higher proportion in fatty tissue of mammary gland; therefore, IGF-1 synthesis is closely related to fat matrix of gland (Purup et al., 2000). Considering the close relation of IGF-1 and cell mitogenesis, it can be assumed that PAR increase would stimulate, indirectly, epithelial growth by increasing IGF-1 synthesis (Purup et al., 2000).

For IGFR1, higher mRNA expression in HG (P<0.001) and LG (P=0.002) than MA on day 42 was observed. However, on day 84 these differences (P>0.05; Figure 5B) were notdetected and mRNA expression was lower (P<0.001) than the values observed on day 42. Within IGF-1 system, IGFR1 is responsible for initiating the IGF-1 pathway by interaction with IGF-1 or insulin (Byun et al., 2012). An increase on mRNA abundance of IGFR1 may indicated indirectly higher IGF-1 action, once a greater site to binding are available.

Regarding of mRNA abundance of TGF-βR1, we observed that HG treatment showed values higher than LG (P<0.001) and MA (P=0.005) on day 42, while no difference (P>0.05) was observed on day 84 (Figure 5C). The actions of TGF-*β1* are mediated by binding to highly specific cell surface receptors of which most cells have three types (I, II and III) (Roberts and Sporn, 1990). We investigated type I receptor, once it is directly involved in signal transduction. Purup et al. (2002) showed an extensive staining for type I receptor in ductal epithelium promoting an effect on TGF-*β1*. TGF-*β1* affects the proliferation of epithelial tissue, once it is involved in terminal points' regression of epithelium tissue, thus demonstrating its potential inhibitory effect on mammary epithelium growth (Barcellos-Hoff, 2011). In mammary gland of rats, increased expression of TGF-*β1* gene during puberty extremely reduced ductal growth rate as well as the expansion of ductular structure (Akers,

2006). In in vitro systems, an increased concentration of TGF-*β1* in cell cultured medium inhibited DNA synthesis, achieving 66% of inhibition on DNA synthesis (Purup et al., 2000). Moreover, Sejrsen et al. (1998) using two group of Holstein heifers (from 90 or 250 kg of BW), and moderate (ADG = 700 g/d) or high performance (ADG = 1,150 g/d), observed that concentration of TGF-*β1* on serum was significantly higher (23.3 vs 15.8 ng/mL) in heifers at the high feeding level than in those at the moderate feeding level.Therefore, although based on mRNA expression, our results strongly suggest that TGF-*β1* may be involved in inhibition of mammary growth and development in heifers.

In general, gene expression at day 84 was lower than day 42, however, at this point, there is no available result in the literature that could enlighten this fact. Perri et al. (2014) also observed a temporal variation in IGF-1 and estradiol during development of heifers. Therefore, further studies are needed to understand the performance effect on gene expression variation over time.

In summary, the higher mRNA expression of IGF-1 and IGF-1Rof heifers in continuous growth (HG and LG) when compared to restricted feeding animals (MA) e may indicate that undernutrition impairs mammary gland development, but with no difference between performance levels (HG and LG). However, it was also observed that high performance heifers (HG) were more likely to reduce mammary epithelium development compared to moderate performance (LG) when assessing TGF-*β*R1 gene expression. Gene expression results confirm the chemical and physical results, whichindicate that animals in high performance show greater fat accumulation in mammary gland, which in turn inhibits the full growth of epithelium.

A second explanation for reduction of epithelial development in high performance crossbred heifers may be linked to a higher secretion of leptin in mammary glands of heifers with higher energy consumption (Table 3). In a parallel study, Weller et al.(2016) observed higher gene expression of hormones related to leptin synthesis in gland of HG heifers. Adipocyte-derived hormone leptin illustrates the amount of energy stored in adipose tissue, which is affected by nutritional status (Matarese et al., 2005). Leptin affects cell proliferation induced by IGF-1 (Smith et al., 2002) and stimulates macrophages increase, which may influence mammogenesis by inhibiting IGF-1 signaling. For instance, after stimulation with exogenous leptin, it was detected an increase of macrophage secretion in rodents, which will increase their phagocytic activity and cytokinins production (Loffreda et al., 1998; Matarese et al., 2005), which was previously associated with inhibition of cellular proliferation induced by IGF-1 in other types of cells (Shen et al., 2004).

Although the amount of fat in mammary gland have been linked with reduced parenchymal development in extensive literature review, Hovey (2010) clearly showed the need of adipocytes from stroma for full parenchymal development. For example, fatty acid deficiency in rodent diet affected alveolar development (Knazek et al., 1980; Miyamoto-Tiaven et al., 1981). Thus, an optimal amount of fat range in mammary gland seemed to be needed as adequate substrate for stimulating growth factors synthesis to trigger the full development of the epithelial tissue.

Given the above, this range may be related to an intermediate performance between LG and HG. Therefore, the proposed hypothesis on which the heifer under high performance on prepubertal has not impaired the parenchymal development when fed with adequate energy-protein balance in the diet was not accepted. This performance range seems to be related to the amount of fat deposited in mammary gland, which although necessary for PAR development, impairs epithelium growth if in excess.

CONCLUSION

In conclusion, the performance level on prepubertal crossbred heifers influences composition of mammary gland, independent of energy protein ratio on diet, where HG heifers showed a greater amount fat and less protein on PAR tissue. The highest pixel values found on HG ultrasound images confirm the fat accumulation on PAR in function of performance. In addition, heifers in HG and LG had similar mRNA expression forIGF-1 on PAR, while a greater mRNA expression forTGF-βR1was observed on PAR of HG, indicating a potential reduction of IGF-1 mitogenic effect. The performance level reduce the epithelial area on PAR and increase the adipocyte area on PAR. Although the literature results are controversial, in this work we have showed that performance alone is responsible for reducing the parenchymal development. However, additional works are needed to prove this effect will be expressed in future lactation.

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Table 1. Ingredients and chemical composition of ration and diet.

Ingredients	Ration	Diet
	% DM	
Corn silage	-	59.2
Corn	54.6	22.3
Soybean meal	23.0	9.4
Soybean meal By Pass	18.8	7.7
Urea	0.7	0.3
Minerals[1]	2.8	1.2
Composition		
DM (g/kg fresh matter)	889.8	331.7
Mineral (g/kg DM)	48.7	60.3
CP (g/kg DM)	270.0	171.2
RDP (g/ kg DM)	144.6	109.1
RUP (g/kg DM)	126.4	62.1
NDF (g/kg DM)	145.2	361.8
EE (g/kg DM)	28.1	21.3
NFC (g/kg DM)	511.9	390.1
TDN (g/kg Dm)	806.1	711.2

[1] limestone – 40 g/kg; dicalcium phosphate – 15 g/kg; sulfur flower – 0,5 g/kg; Potassium iodate – 4 mg/kg; sodium selenite – 1 mg/kg; cobalt sulfate – 1,5 mg/kg; copper sulphate – 60 mg/kg; manganese sulphate – 5 mg/kg; zinc sulphate – 0,2 mg/kg.

Table 2. Gene names, primers pairs sequence and amplification efficiency of each gene

Genes[1]	Accession number[2]	Primer sequence (5'-3')	BP[3]
TGFβR1	NM_174621.2	F-TGGGACTAGTATTCTGGGAAGTA R- CTGATGGATCGGAAGGTACAAG	102
IGF1R	NM_001244612.1	F-GTATGGAGGAGCCAAGCTAAA R-GTCTTGGCCTGAACGTAGAA	123
IGF1	NM_001077828.1	F- AGCAGTCTTCCAACCCAATTA R-ACAGGGCCAGATAGAAGAGA	103
HPRT1	NM_001034035.2	R-GTGGGATATGCCCTTGACTATAA F-GGACTCTCATCTTAGGCTTTGT	104
RSP15A	NM_001037443.2	F-GGAGTGATCAGCCCTAGATTTG R-AGCTGAGGTTGTCAGTACAATG	108

[1]Genes: transforming growth factor beta receptor 1 (TGFβR1), caspase 3 (CASP3), insulin-like growth factor 1 receptor (IGF1R), insulin-Like growth factor 1 (IGF1),hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein S15a (RSP15A).[2]Accession number in Genbank (htpp://www.ncbi.nlm.nih.gov).[3]Amplification size in base pairs.

Table 3. Comparative analysis of intake and nutritional requirements based NRC, 2001 and values obtained in the experiment for heifers in ad libitum intake.

	NRC, 2001		Experiment[1]
Average body weight (kg)	130	143	143
DMI (kg)	3.80	4.000	3.980
CP (kg/d)	0.683	0.686	0.696
RDP (kg/d)	0.438	0.440	0.426
RUP (kg/d)	0.245	0.246	0.270
MP (kg/d)	0.468	0.470	0.450
ME (Mcal/d)	8.700	9.400	10.16

MP – metabolizable protein, estimated according NRC (2001); EM – metabolizable energy, calculated TDN * 0.04409 * 0.82 NRC (2001); RDP = rumen degradable protein, calculated by TDN x 1.18, according NRC (2001); RUP – rumen undegradable protein.

[1] Values obtained of HG treatment.

Table 4. Evaluation of weight gain and growth of body measures

	Treatments			SEM	P value
	HG	LG	MA		
Initial BW	105.00	104.33	98.16	60.80	0.721
Final BW	181.20	146.33	107.00	7.53	< 0.001
Intake					
DM	3.98	2.56	1.48	0.758	< 0.001
OM	3.76	2.42	1.40	0.758	< 0.001
CP	0.69	0.43	0.25	0.320	< 0.001
NDF	1.41	0.94	0.55	0.449	< 0.001
NFC	1.58	0.99	0.57	0.488	< 0.001
Body measures					
ADG (kg/dia)	0.907 a	0.500 b	0.105 c	0.027	< 0.001
BLG (cm/dia)	0.218 a	0.069 b	0.083 b	0.040	0.012
HGG (cm/dia)	0.166 a	0.065 b	-0.039 c	0.011	< 0.001
WHG (cm/dia)	0.081 a	0,053 ab	0.029 b	0.013	0.004

BLG = body length growth; HGG = heart girth growth; WHG = withers height growth.Lower letters indicate differences (P < 0.05).

Table 5. Chemical and physical evaluation of the mammary gland and body composition of heifers according to the treatments

	Treatments			SEM	P value
	HG	LG	MA		
Slaughter weight (kg)	181.20	146.33	107.00	7.53	< 0.001
physical assessment of the mammary gland					
MG (kg)	1.405 a	1.210 a	0.621 b	0.111	< 0.001
PAR (kg)	0.237 b	0.356 a	0.178 b	0.466	0.037
MFP (kg)	1.027 a	0.603 b	0.313 c	0.790	< 0.001
PAR:MG	0.097 a	0.195 b	0.218 b	0.032	0.05
chemical assessment of the mammary gland					
DM (g/Kg MN)	586.1	580.3	554.2	39.35	0.681
Ash (g/kg MS)	7.37	11.55	10.18	1.37	0.132
CP (g/kg MS)	91.9 c	109.7 b	128.7 a	6.90	0.009
EE (g/kg MS)	900.7 a	878.7 b	861.1 b	7.36	0.009
chemical assessment of body composition					
Ash (g/kg MS)	128.9	113.7	131.5	8.80	0.322
CP (g/kg MS)	475.4 b	496.3 b	537.3 a	14.44	0.027
EE (g/kg MS)	395.7 a	390.0 a	331.2 b	15.03	0.015

MG – mammary gland; PAR – parenchymal tissue; MFP – mammary fat pad; PAR:MG – parenchymal tissue weight: mammary gland weight ratio. Lower letter indicate differences (P < 0.10)

Table 1. Serum insulin and estradiol levels from crossbred heifers submitted to different performances

	Treatments				P valeu		
	HG	LG	MA	SEM	T	P	T*P
Insulin (UI/mL)	0.977 a	0.826 a	0.583 b	0.077	0.003	0.001	0.068
Estradiol (pg/mL)	21.417	24.500	23.806	1.489	0.351	0.310	0.962

T – treatment effect; P – period effect; T*P – effect of interaction period*treatment.

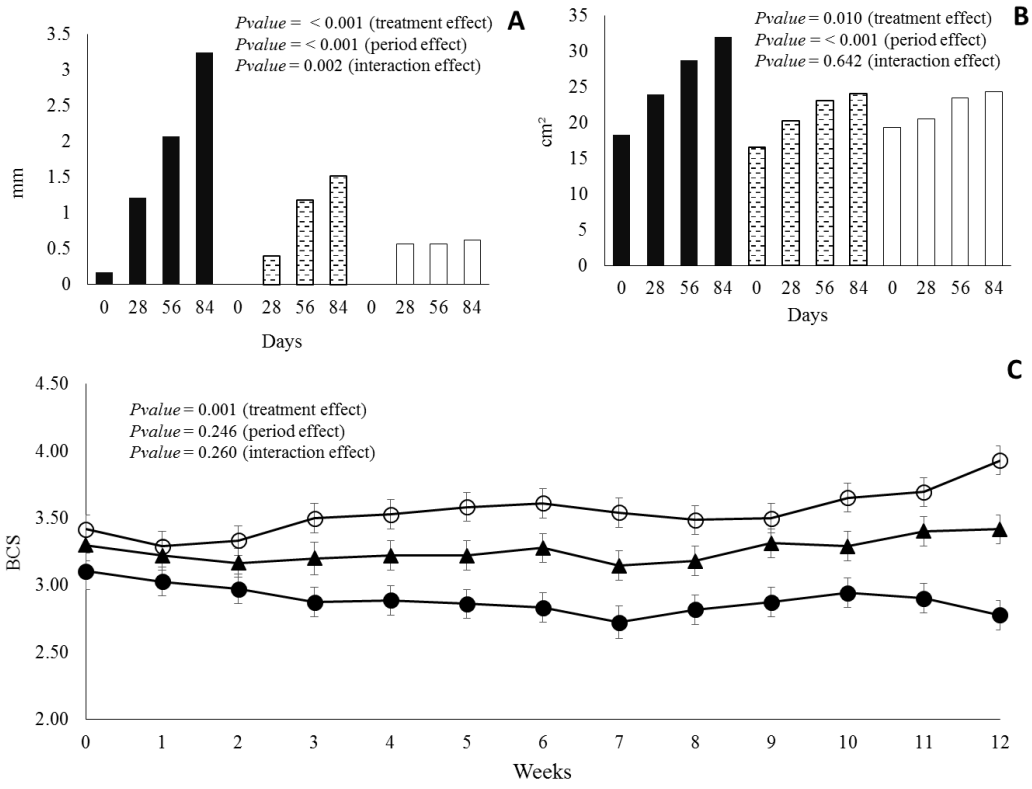

Figure 1. Albino et al.

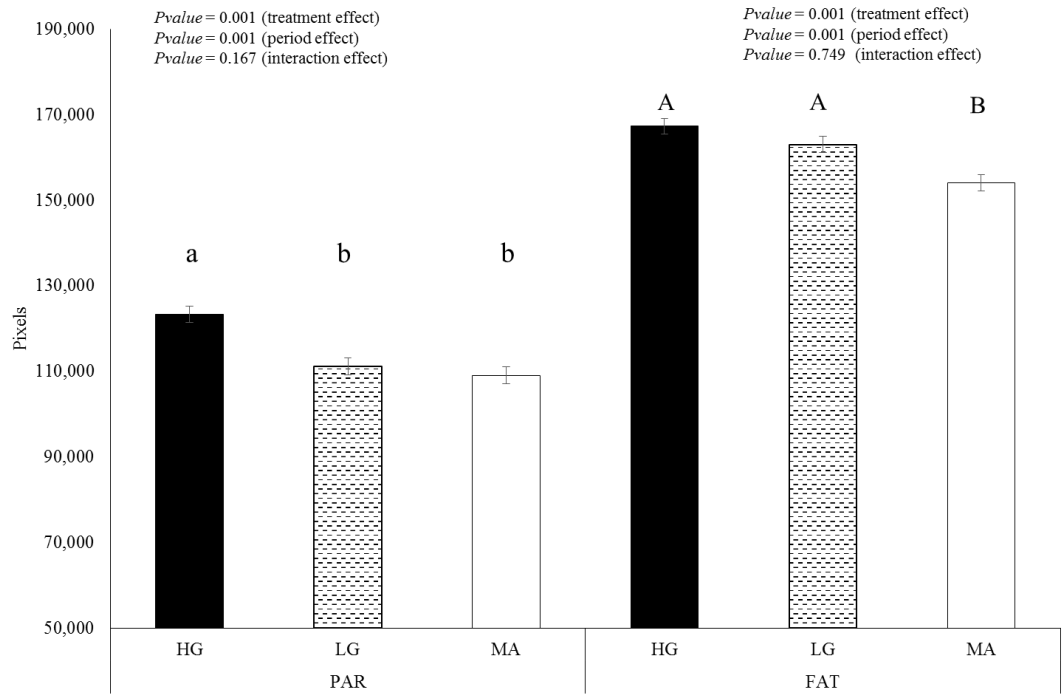

Figure 2. Albino et al.

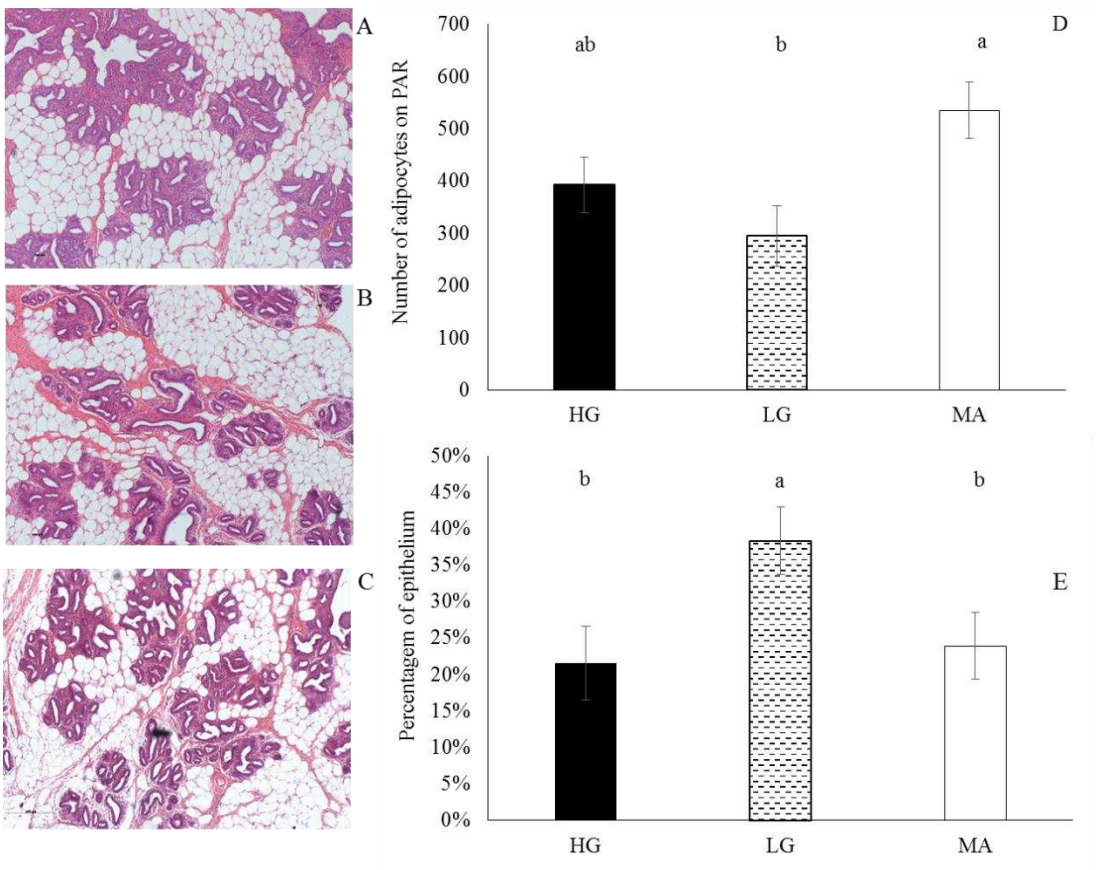

Figure 3. Albino et al.

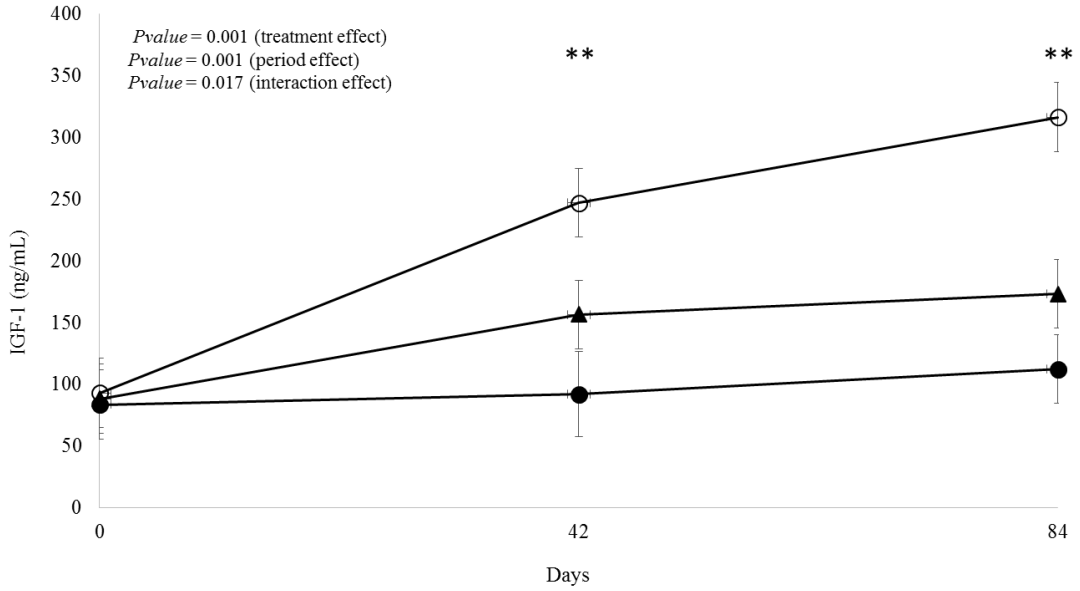

Figure 4. Albino et al.

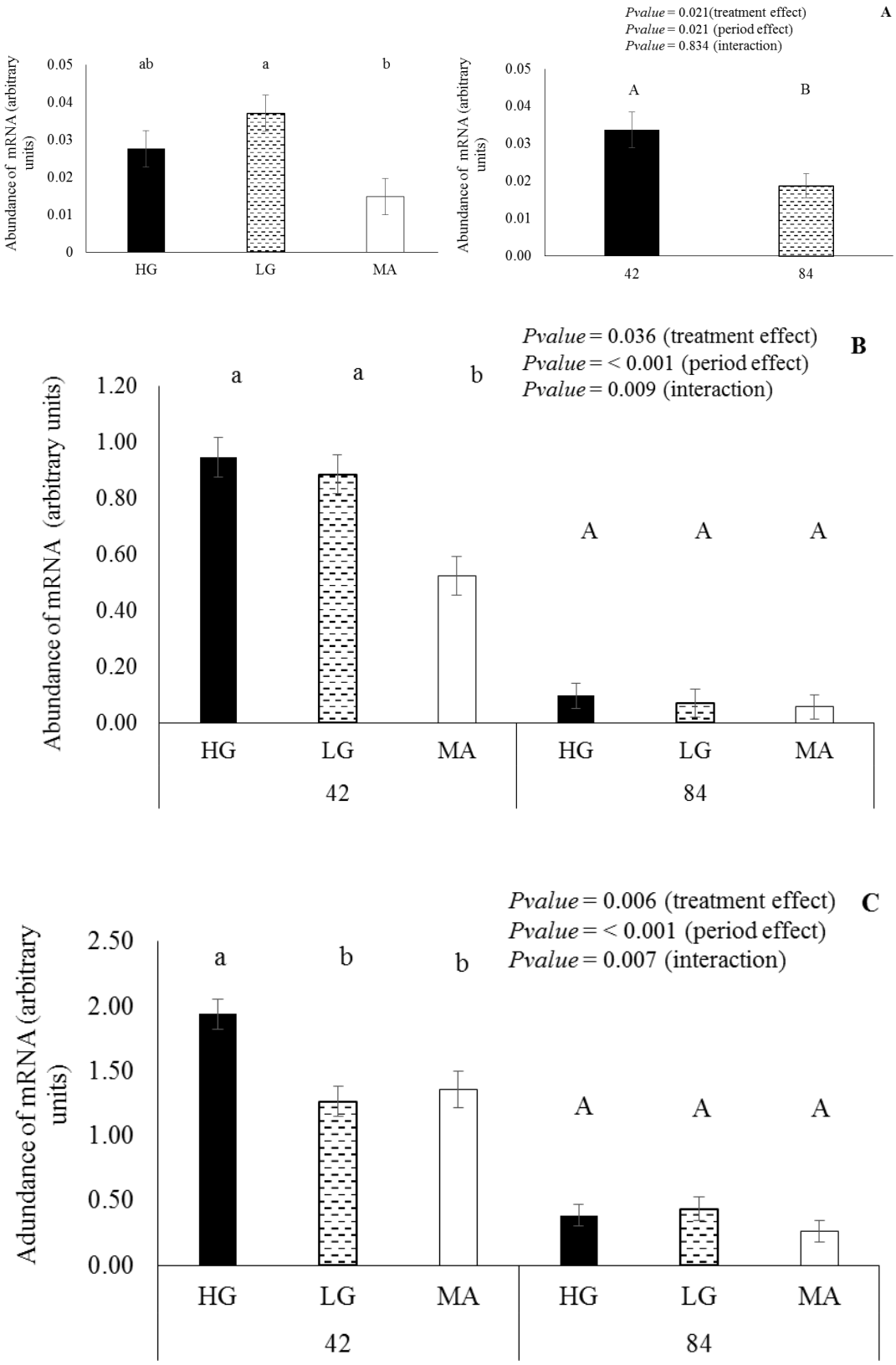

Figure 5. Albino et al.

Figure 1. Subcutaneous fat thickness (A) and rib eye area (B) of heifers over experimental period, for high gain (HG, black column), low gain (LG, crosshatch column) and maintenance (MA, empty column) treatments. Body condition score (C) of the heifers over weeks, for high gain (HG, open circle), low gain (LG, closed triangle) and maintenance (MA, closed circle) treatments.

Figure 2. Pixel values of mammary parenchyma (PAR) and mammary fat pad (MFP) for high gain (HG, filled column), low gain (LG, crosshatch column) and maintenance (MA, empty column) treatments. Lowercase letters indicate difference ($P < 0.05$) for PAR. Uppercase letters indicate difference ($P < 0.05$) for MFP.

Figure 3. Representative images of mammary parenchyma (PAR) for: high gain (A), low gain (B) and maintenance (C) treatments. Number of PAR adipocytes differed by treatment (D), as did percent of PAR occupied by epithelium (E).Lowercase letters indicate differences ($P < 0.05$).

Figure 4. Serum insulin like growth factor-1 (IGF-1) concentration for high gain (HG - open circle), low gain (BG, closed triangle) and maintenance (MA, closed circle) treatments. ** $P < 0.05$.

Figure 5. Mammary parenchymal mRNA abundance of: IGF-1 (A), IGFR1 (B) and TGFRβI (C). Relative abundance of IGF-1 was affected by dietary treatment and period (A). Relative abundance of IFGR1 and TGF-βR1 were affected by the combination of dietary treatment and period, B and C, respectively. Lowercase letters indicate differences ($P < 0.05$) in day 42 or between periods. Uppercase letters indicate differences ($P < 0.05$) in day 84 or between periods.

CHAPTER 2

TECHNICAL NOTE: MAMMARY ULTRASONOGRAPHY

Interpretive Summary

Technical Note: Mammary Gland Ultrasonography to Evaluate Mammary Parenchymal Composition in Prepubertal Heifers. By Albino et al. Ultrasonography of mammary parenchyma (PAR) has an accuracy from 0.77 to 0.93 to predict the PAR composition in prepubertal heifers. This is an important advancement in non-invasive monitoring of mammary development and may soon be used on dairy farms to monitor composition of PAR growth under various management scenarios.

Technical Note: Mammary Gland Ultrasonography to Evaluate Mammary Parenchymal Composition in Prepubertal Heifers

R. L. Albino*, S. E. F. Guimarães*, K. M. Daniels†, M. M. S. Fontes*, A. F. Machado*, G. B. dos Santos‡, M. I. Marcondes*[1]

*Universidade Federal de Viçosa,Viçosa, MG, Brazil, 36570-900

†Dairy Science Department, Virginia Polytechnic Institute and State University, Blacksburg 24061

‡Programa de Pós Graduação em Clínica e Reprodução da Universidade Federal Fluminense, Niterói, RJ, Brazil, 24230340

[1]Corresponding author: marcosinaciomarcondes@gmail.com

ABSTRACT

Bovine mammary gland development studies are often terminal or involve invasive biopsy procedures. Therefore, non-invasive means of assessing mammary development should be considered as alternative methods in live animals. The objective was to test if mammary ultrasonography can be used as a noninvasive way to estimate mammary parenchyma (**PAR**) composition in prepubertal dairy heifers with different average daily

body weight gains. In the 84 d preceding, the ultrasound exam heifers were maintained in one of three treatment groups. Individual heifers were fed either a high gain (**HG**; 1 kg/d; n=6), low gain (**LG**; 0.5 kg/d, n=6), or maintenance (**MA**, n=6) treatment diet. To achieve desired body weight gains, heifers were fed differing amounts of the same silage-based diet. Mammary glands of eighteen crossbred heifers Holstein:Gyr (H:G) underwent a single mammary ultrasound exam immediately before heifer slaughter, which took place when heifers weighed 142.0 ± 8.0 kg and were 200 d old. The 4 mammary glands of each heifer were evaluated using a real-time B-mode ultrasound machine equipped with a 6.5 MHz micro-convex transducer. Digital images (8-bit) of glands were obtained and PAR was identified within gland. Average pixel values per unit of PAR area were determined for each gland and analyzed at the level of heifer. Pixel results were interpreted on the basis that lower average pixel values reflect PAR with relatively high amounts of protein as opposed to fat. To help validate that pixel value within PAR is associated with composition of PAR, pixel findings were compared to histological (number of adipocytes in PAR (**Nad**); epithelial area in PAR (**Ep**)) and biochemical (percent crude protein in PAR (**%CP**); percent ether extract in PAR (**%EE**); PAR weight (**WPAR**), mammary fat pad weight (**WFAT**)) composition of PAR in these same heifers. Within PAR, %EE and WFAT were positively correlated with pixel values, while %CP, Ep and Nad were negatively correlated. Parenchyma weight did not correlate with pixel values. Regression analyses (fixed effect log-pixel value; random effect treatment) were used to estimate: Nad, Ep, %CP, %EE, WPAR and WFAT. Sensitivity analysis of regression equations revealed that accuracy of tested equations ranged from 0.77 to 0.93 and the precision ranged from 0.56 to 0.82. Concordance correlation coefficients of the equations ranged from 0.41 to 0.76. In conclusion, ultrasonography of PAR can accurately measure and predict PAR composition in prepubertal dairy heifers growing at various rates of gain.

Key Words: dairy heifer, ultrasound, mammary gland

Background

Mammary glands consist of two major types of tissues that serve different functions. As summarized by Esselburn et al. (2015), these are: 1) epithelial tissue and surrounding stromal elements, collectively called mammary parenchyma (**PAR**), and 2) stromal tissue that lies adjacent to PAR and does not contain epithelial structures, and instead mainly consists of connective tissue structures such as adipose. For this reason, mammary stroma is often referred to as mammary fat pad (**MFP**).

Understanding of growth dynamics of these mammary tissues as well as the influence of nutrition on mammary gland development have been the subject of study over the years (Sejrsen et al, 1981; Brown et al. 2005, Meyer et al., 2006). Esselburn et al. (2015) recently showed that in heifers younger than 2 mo of age, obtaining weekly PAR measurements via ultrasound was an effective quantitative tool for measuring changes in PAR area in vivo. Although estimation of PAR composition via ultrasonography was not an experimental objective of Esselburn et al. (2015), those authors noted that such estimation is feasible. That was the motivation for our current work and is a distinguishing feature of this study

Ultrasound has been widely used in the meat industry to monitor both subcutaneous fat thickness and intramuscular fat accumulation (Williams, 2002). The measurement of intramuscular fat accumulation in carcasses is possible due to differences in the way that fat and protein reflect sound waves in body tissues (Brethour et al., 1990).

Our hypothesis was that mammary ultrasonography can be used as a noninvasive way to estimate PAR composition in prepubertal dairy heifers with different average daily gains.

Methodology

The study was conducted in the Animal Science Department of the Universidade Federal de Viçosa (DZO-UFV), Viçosa-MG. The institutional ethics committee approved all procedures (protocol number, 20/2015). Eighteen crossbred heifers Holstein:Gyr (H:G) were used in this experiment; they were part of a larger experiment that dealt with effects of nutrient intake on mammary growth and composition. Full experimental details are reported elsewhere (Weller et al., 2016, under review, JDS-16-11532). Briefly, heifers were allotted to one of three dietary treatments when they were 3 to 4 mo of age (body weight, 102.2 ±3.4kg). Treatments were: high gain (**HG** (n = 6); 1.0 kg/day BW gain), low gain (**LG** (n = 6); 0.5 kg/day BW gain) and maintenance (**MA** (n = 6); (0.0 to 0.1 kg/d BW gain). The MA treatment was kept slightly above 0.0 kg/d ADG to avoid mobilization of body tissues. To achieve desired body weight gains, heifers were fed differing amounts of the same silage-based diet. Amount of ration offered was adjusted every 2 wk based on heifer BW gains. Heifers were housed and fed individually; the experimental unit was heifer.

At the end of the 84 d experiment, heifers were slaughtered (140.0 ± 8.0 kg and 200 d old) in order to assess body and mammary composition (Weller et al., 2016, under review). Relevant dependent variables reported by Weller et al. (2016), and also used here include: number of adipocytes in PAR (**Nad**); epithelial area (**Ep**); percent crude protein of PAR (**%CP**); percent ether extract of PAR (**%EE**); PAR weight (**WPAR**), and mammary fat pad weight (**WFAT**) in these same heifers. For full description of methodology on these 6 dependent variables, the reader is directed to Weller et al. (2016).

The 4 mammary glands of each heifer were evaluated in a single ultrasound exam prior to slaughter using a real-time B-mode ultrasound machine equipped with a 6.5 MHz micro-convex transducer (DP2200, Mindray, China). Heifers remained standing for the ultrasound exam; sedatives were not used. Commercial acoustic gel (Chattanooga Group,

Inc., Chattanooga, TN) was applied to each gland prior to ultrasounding. A single operator performed all ultrasound exams.

With the heifer restrained in a standing position, the lubricated micro-convex transducer was applied to the base of each teat at a 45° angle, in a caudal-to-cranial placement (Figure 1A). This procedure was obtained from Nishimura et al. (2011) and adapted (Albino et al., 2015).

Still image capture software available on the ultrasound machine was used to capture two 8-bit digital images for each gland (Figure 1B). These images were visually inspected and the image with the best PAR definition was chosen; subsequent evaluations were done based on 4 images per heifer.

The PAR within each digital image was evaluated using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). PAR is hypoechoic (black) on ultrasound when compared to MFP (Esselburn et al., 2015), which is hyperechoic (white). Therefore, the most hypoechoic region of each image was identified as PAR (Figure 1B). Within the area identified as PAR (3 squares each measuring 0.4 cm^2)were randomly overlayed onto the image in ImageJ (Figure 2).

Finally, an average pixel value per unit area was determined for each square using a conversion factor in ImageJ (1 cm = 50 pixels). In 8-bit images, each pixel is numerically represented on a scale of 256,000 shades of gray (0 = black; 255,000 = white) according to their brightness (Ferreira & Rasband, 2011). Therefore, an area of 0.4 cm^2 with an average pixel value of 100,000 is blacker (more hypoechoic) than a similar square with a value of 200,000. Further, this variation of gray shades is related to reflection capacity of sound waves imparted by the tissue. Fat tissue has greater reflectivity of sound waves, producing more hyperechoic or white images. On the other hand, protein has lower reflectivity thereby producing hypoechoic or blacker images (Brethour et al., 1990). Thus, squares with lower

average pixel values should reflect PAR with relatively high amounts of protein as opposed to fat. Finally, to match mammary composition data, which were collected at the level of udder, the 12 average pixel values per heifer were summed and divided by twelve.

Statistical Analyses

Pixel data from mammary glands were log transformed prior to statistical analysis to satisfy rules of analysis of variance (Davis, 1989, data not shown).

To evaluate the relationship between dependent variables a regression analysis was used that included the fixed effect of log of pixel values and the random effect of treatment (HG, LG, MA) to estimate: Nad, Ep, %CP, %EE, WPAR, and WFAT using PROC MIXED (SAS 9.3, 2011).

Subsequently, a sensitivity analysis was performed by cross-validation (Davison and Hinkley, 1997) to estimate the mean square error prediction (MSEP), correlation coefficient (ρ), accuracy (C_b), coefficient of determination (r^2), and concordance correlation coefficient (CCC) of each empirical equation using R statistical software (R Development Core Team, 2009). The procedure was repeated 1,000 times, and means of MSEP, CCC, ρ, C_b, r^2 are shown in Table 1.

Findings

The percentage of ether extract (%EE) in PAR was positively ($r = 0.318$, $P = 0.024$) correlated with pixel value in PAR. This was as expected because high pixel values are associated with image whiteness, which equates with fat here. In agreement, crude protein percent of PAR (%CP) was negatively correlated ($r = 0.467$, $P = 0.006$) with pixel value.

No relationship was observed between pixel value and weight of PAR (WPAR) ($P = 0.645$). We believe that the high animal to animal variation in WPAR may have masked any effect. A positive linear relationship was identified between pixel value and WFAT ($r = 0.596$, $P = 0.013$), which indicates a similar effect as observed in %EE noted above (Table 1).

Similarly, relationships between pixel value and Ep ($r = 0.529$, $P = 0.001$) and Nad ($r = 0.672$, $P < 0.001$) were both identified as negative, that is, as pixel value increased epithelial tissue area in PAR (Ep) and adipocyte number in PAR (Nad) decreased. This result is consistent with PAR composition that is mainly formed by epithelial and adipose tissue (Weber et al., 2000). The negative relationship between pixel value and Nad is explained by the low deposition of triglyceride within PAR adipocytes of animals with restricted energy consumption (in this case, MA), which leads to a lower area per adipocyte but a higher number of adipocytes per unit area (Azain, 2003). Thus, within similarly sized microscopic fields of view, a higher total number of PAR adipocytes was observed in MA compared to HG and LG, and these adipocytes contained less accumulated fat as evidenced by lower %EE in MA treatment (data not shown).

One drawback of our technique worth mentioning is that our ultrasound images did not allow us to distinguish between epithelial duct tissue and duct lumens within PAR – both were observed as hypoechoic (black) features in comparison to the surrounding mammary fat.

From the sensitivity analysis, we observed that 75% of the average error prediction was related to random effects (MaF), possibly not controlled by the model, which in turn indicates a small proportion of error associated with the systematic error (MoF) and bias (SB) of models estimates. The MoF is concerning the direction of the curve predicted and SB is related with slope of linear regression, being both associated with prediction problems of model.

Equation precision varied from 0.56 to 0.82, indicating that the models have limitations to properly predict similar values (Tedeschi, 2006). On the other hand, the models presented medium to high accuracy (0.772 to 0.938), showing the ability to predict the real mean values of pixels (Tedeschi, 2006). The CCC, which indicates the precision and

accuracy of the technique, was not high, however, it is noteworthy that the number of observations used to establish the regression equations was low (n=18), which made it hard to increase the technique precision, and directly affected the CCC (Table 1). However, even considering a small number of replications, the pixel values in PAR were correlated with 5 of 6 variables tested. Therefore, it may be inferred that ultrasound is an acceptable tool to assess and predict mammary gland composition in prepubertal heifers.

Conclusions

Ultrasonography of PAR can accurately measure and predict PAR composition in prepubertal heifers growing at various rates of gain. This is an important advancement in consistency of non-invasive monitoring of mammary development and may soon be used on dairy farms to monitor composition of PAR growth under various management scenarios. However, before large-scale implementation, more studies involving greater numbers and sizes of animals are suggested to increase technique precision.

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Supplementary material. Effects of 84 d of exposure to either a high-gain, low-gain, or maintenance diet on mammary measurements in prepubertal Holstein x Gyr heifers

Variable	Treatment[1]			SE	P value
	HG (n = 6)	LG (n = 6)	MA (n = 6)		
Pixel value[2]	123,337[a]	111,126[b]	109,064[b]	1,963	< 0.001
%EE[3]	90.07[a]	87.87[ab]	86.11[b]	0.73	0.008
%CP[4]	9.19[b]	10.97[ab]	12.87[a]	0.69	0.009
WFAT, g[5]	1,405[a]	1,210[a]	0.621[b]	11.9	<0.001
WPAR,g[6]	237.3[b]	356.8[a]	178.2[b]	4.6	0.037
Ep[7]	21.54[a]	38.30[b]	23.91[a]	4.8	0.051
Nad[8]	392.73[a]	294.89[a]	535.39[b]	5.6	0.027

[1]Treatments were: high gain (**HG**; 1.0 kg/day BW gain), low gain (**LG**; 0.5 kg/day BW gain), or maintenance (**MA**; (0.0 to 0.1 kg/d BW gain). Heifers were allotted to treatments when they were 3 to 4 mo of age (body weight, 102.2 ±3.4kg). To achieve desired body weight gains, heifers were fed differing amounts of the same silage-based diet. Treatments were administered for 84 d, at which point heifers underwent a single mammary ultrasound exam prior to slaughter. Rows with different superscripts differ at $P < 0.05$.

[2]Pixel value = average pixel value of PAR. 0 = black (hypoechoic); 255,000 = white (hyperechoic). Fat tissue is known to have relatively greater reflectivity of sound waves, producing more hyperechoic or white images.

[3]%EE = percent ether extract in PAR, originally reported in Weller et al. (under review).

[4]%CP = percent crude protein in PAR, originally reported in Weller et al. (under review).

[5]WFAT = weight of mammary fat pad, originally reported in Weller et al. (under review).

[6]WPAR = weight of PAR, originally reported in Weller et al. (under review).

[7]Ep = percent of PAR occupied by epithelial cells, originally reported in Weller et al. (under review).

[8]Nad = number of adipocytes in PAR per unit area, originally reported in Weller et al. (under review).

Table 2. Sensitivity analysis of parenchymal components of dairy heifers estimated by ultrasonography

Item[1]	EE%	CP%	WFAT	WPAR	Ep	Nad
β_0	-0.001 ± 0.35	1.38 ± 0.39	-14,287 ± 4,689	-516.39 ± 1609	457.27 ± 105.61	11,338 ± 1,705
β_1	0.078 ± 0.03	-0.11 ± 0.03	1,321 ± 414	66.67 ± 141	-37.70 ± 9.28	-948.59 ± 149
P-value	0.024	0.006	0.013	0.645	0.001	< 0.001
MSEP	0.0003	0.0002	43,658.27	7,719.63	28.17	6,887.93
Partition of MSEP, %						
Mean Bias (MoF)	7.18	7.89	8.94	7.87	8.19	9.36
Systematic Bias (SB)	6.73	10.90	15.15	6.76	14.35	10.33
Random Error (MaF)	86.08	81.20	75.91	85.36	77.45	80.31
CCC, range 0 to 1	0.413	0.555	0.687	0.015	0.615	0.764
ρ	0.564	0.684	0.772	0.053	0.727	0.820
C_b	0.773	0.829	0.906	0.271	0.914	0.938
r^2	0.318	0.467	0.596	0.073	0.529	0.672

[1]EE = percentage of ether extract in mammary parenchyma (PAR); CP = percentage of crude protein in PAR; Weight FAT = weight of extra-parenchymal fat (g); Weight PAR = weight of PAR (g); Ep = area occupied by epithelial tissue in PAR; Nad = number of adipocytes in PAR. MSEP = mean square error prediction; ρ = correlation coefficient; C_b = accuracy; r^2 = coefficient of determination; CCC = concordance correlation coefficient.

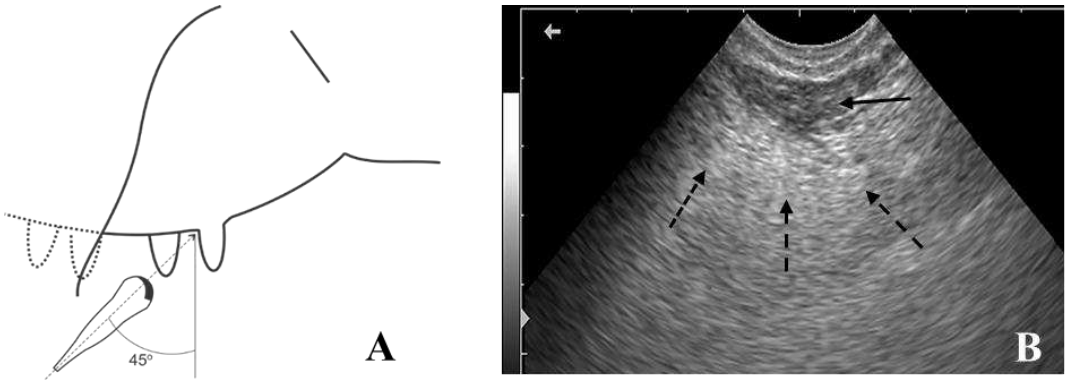

Figure 1. Albino et al.

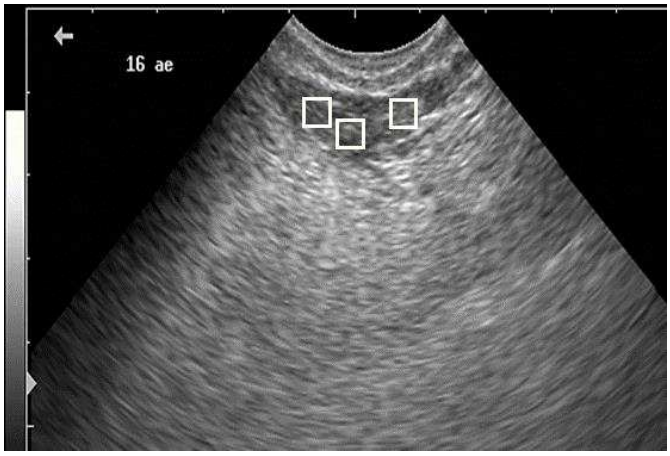

Figure 2. Albino et al.

Figure 1. Drawing of the probe position at the heifer's mammary gland (Albino et al., 2015) (A) and ultrasound image (B) representing the mammary fat pad (MFP) by dashed arrows and parenquimal area (PAR) by filled arrows.

Figure 2. Demonstration of square positioning used to evaluate pixel value in mammary parenchyma (PAR) with ImageJ. Each square is 0.4 cm^2.

CHAPTER 3

CASE REPORT: Mammary gland biopsy in young heifers

RL Albino[a], JT Silva[a], LP Rocha[a], VCL Morais[a], MMDCastro[a], FAC Villadiego[b], SEF Guimarães[b], MI Marcondes[a*]

[a]Animal Science Department, Universidade Federal de Viçosa, Minas Gerais, Brasil.

[b]Veterinary Department, Universidade Federal de Viçosa, Minas Gerais, Brasil.

ABSTRACT.

Biopsy is a feasible method whereby it is possible to take tissue samples without the need to slaughter. The parenchymal tissue study allows us to understand the influence of performance under mammary gland development. The gene expression of mammary gland parenchyma obtained through biopsy showed proper mRNA abundance from samples.

Key words: parenchyma, mammary gland, biopsy

RESUMEN.

La biopsia es un método donde es posible tomar muestras de tejido sin necesidad de sacrificar el animal. El estudio del parénquima permite entender la influencia del desempeño sobre el desarrollo de la glándula mamaria. La expresión génica del

*corresponding author: marcosinaciomarcondes@gmail.com

parénquima de muestras obtenidas mediante biopsia mostro adecuado abundancia RNAm.

Palabras clave: parénquima, mama, biopsia.

Biopsy has been a technique used over the years to study in detailthe systems that control milk synthesis and mammary gland development (Knight et al 1992). Among its advantages, we should highlight the ease of the procedure and absence of slaughter, thus enabling several analyses of the same animal (Murugaiyahet al 1999).

Knight and Peaker (1984) were able to quantify a representative sample of parenchymal tissue obtained through a biopsy procedure. Marx and Caruolo (1963) took samples with a scalpel and showed that this technique does not limit the sample size, as is observed with needle biopsy (Stelwaeen and Grieve 1990). Further, a reduction in recovery time was observed for animals subjected to a scalpel biopsy procedure in relation to needle biopsy (Hibbert 1964).

Many studies have attempted to understand mammary gland development. Most used slaughter as the primary means of studying mammary gland composition. This approach makes it impossible to study first lactation milk production (Meyer et al 2006, Davis-Rincker et al 2008,Albino et al 2015). Thus, we aimed to demonstrate the complete procedure of biopsy used to obtain representative samples of the mammary parenchyma in growing heifers.

The study was conducted at the Animal Science Department of Universidade Federal de Viçosa, Viçosa, MG. This research is part of a work conducted by Weller et al (2016) named“Effects of plane of nutrition on prepubertal heifers mammary gland development”. Briefly, over 84 days, eighteen crossbred Holstein:Gyrheifers, with an initial body weight of 102.2 ± 3.4 kg, and aged between 3 and 4 months, were experimentally evaluated according to protocols approved by the institutional ethics committee (protocol nº 20/2015). Heifers were divided into three treatments, high gain (1.0 kg/day, n = 6), low gain (0.5 kg/day, n = 6), and maintenance (0.1 kg/day, n=6). Mammary gland parenchymal tissue (PAR) growth was studied through samples obtained by biopsy. The biopsy was conducted on the left hindquarter on day 0 and on the right hindquarter on day 42. Three heifers per treatment were chosen randomly for biopsy procedures. At the end of the experiment (day 84) all heifers were slaughtered and samples from PAR were taken (Welleret al2016).

Before beginning the biopsy, the entire surgical environment was sterilized with 10% active chlorine. A table was cleaned with 70% alcohol, sterilized with RNase

Exterminator[1] and installed in the surgical room to facilitate the handling of surgical instruments. RNase Exterminator was used to avoid contamination and degradation of PAR mRNA samples.

After 12h of fasting, an anesthetic base xylazine hydrochloride[2] (1 mg/ 100 kg body weight) was administered intramuscularly. At the first signs of sedation, the heifer was contained and lying on the floor. Then, the hindquarter was trichotomized and disinfected with alcohol 70%. Anesthetic base lidocaine 2%[3] (15 mL per quarter) was administered subcutaneously around the target area (figure 1a). Finally, a 5-cm incision was made with a scalpel in the udder skin. The subcutaneous tissue was opened, permitting access to PAR (figure 1b). Approximately 50 mg of PAR tissue was taken and immediately immersed in the proper tube contain 5mL RNA holder[4]. After sampling, the internal tissues were sutured with catgut number 2 and then the udder skin was sutured with monofilament nylon 3-0. The time spent from the first anesthetic until the end of the procedure was 25 min.

At the first three days after biopsy, an anti-inflammatory base diclofenac sodium[5] (1 mL/50kg BW) was administered every 24 h. Simultaneously, over seven days, the quarter sampled was cleaned with an aseptic solution base chlorhexidine 0.5%, followed by antibiotic and healing topical ointment[6] and spray base silver sulfadiazine[7].

The integrity of samples obtained by biopsy can be evaluated by the absorbance ratio of RNA (absorption at 260 nm) to protein (absorption at 280 nm). The observed ratio of 1.87 ± 0.004 (mean ± standard error) indicates that the level of protein contamination in the sample was low, as pure samples show a ratio between 1.8 and 2.1 (figure 2).

With respect to the animals' welfare, 14 days after the biopsy (data not shown), heifers showed no difference in ultrasound image echogenicity when compared with images obtained from other quarters and even from other heifers, thus showing a complete recovery of the mammary gland. Therefore, it is suggested that biopsy of the mammary gland is a safe procedure for both the animal and the integrity of the sampled tissue.

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1 RNase Exterminator, Bio Agency Biotecnologia, São Paulo, Brasil
2 Dorcipec, Vallée S/A Produtos Veterinários, Montes Claros, Brasil
3 Lidovet, Laboratório BravetLtda, Rio de Janeiro, Brasil
4 RNA holder, Bio Agency Biotecnologia, São Paulo, Brasil
5 Vetflogin, Vallé S/A Produtos Veterinários, São Paulo, Brasil
6 UngüentoVallée, Vallé S/A Produtos Veterinários, Montes Claros, Brasil
7 Max Prata, Vansil Indústria Comércio e Representações Ltda, Descalvado, Brasil

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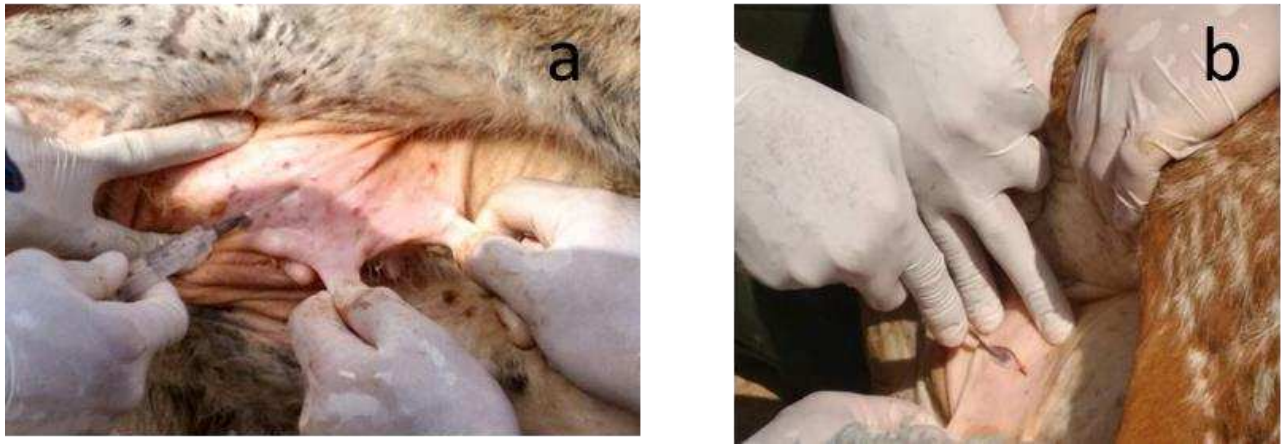

Figure 1. Local anesthetic administration (a). Incision with a scalpel on the outer side of the left hindquarter (b).

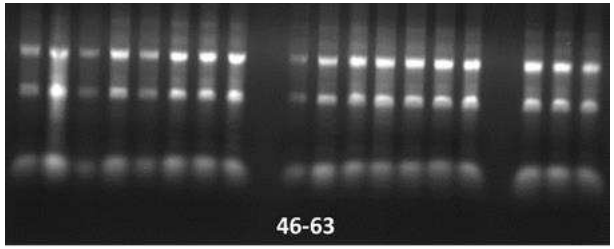

Figure 2.mRNA quality of PAR samples from young dairy heifers.

GENERAL DISCUSSION

The relation between nutritional plan applied during heifers' rearing phase and the ability to express the milk yield potential after calving has encourage several studies over the decades. This continued search of understanding relationships between performance and milk production comes primarily from a complex interaction of nutritional, genetic and hormonal factors that may affect the production, and also from limitations of the techniques used to evaluate the mammary gland development(MGD) during rearing.

In this study, it was possible to isolate the confounding effects of diet and daily weight gain, since the same diet was fed to heifers inweight gains. Thus, we observed that heifers subjected to high performance showed greater accumulation of adipose tissue in the mammary gland, regardless of the diet composition. The higher fat accumulation in the mammary gland was then highly correlated to increased expression of genes that impair the growth of epithelial tissue. Previous studies demonstrated that high weight gains did not impair MGD in Holstein heifers, however this study demonstrated that high weight gain might impair MGD in crossbred Holtein: Gyr heifers. As this was the first study evaluating MGD in crossbred heifers, further researches must be conducted in order to verify whether recommendations obtained from Holstein heifers could be applied in full to crossbred heifers.

Alternative methods to slaughter were tested to monitor continuously and accurately, the mammary gland development ingrowing heifers. Mammary gland evaluations with ultrasound were performed to measure the concentration of pixels in the mammary gland parenchyma. The weight gain affected the pixels concentration

and itwas correlated with biochemical measurements of parenchymal tissue taken after slaughter. These results demonstrated that ultrasound of the mammary gland is a simple, accurate and non-invasive method to monitor the mammary gland development. In addition, it was verified that biopsy of the mammary gland is a safe procedure for both the animal and the integrity of the sampled tissue.