DÉBORA MURATORI HOLANDA

L-ARGININE IMPROVES MAMMARY GLAND VASCULARITY AND ANTIOXIDANT CAPACITY OF LACTATING SOWS

Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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Approved: July 21, 2017.

Márvio Lobão Teixeira de Abreu

Simone Eliza Facioni Guimarães
(Co-advisor)

Alysson Saraiva
(Advisor)

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ABSTRACT

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Lactating sow's mammary glands pass through periods of metabolic burden and oxidative stress. Previous studies have shown that the supplementation of lactating sows with arginine promotes improvements in litter performance, but they do not address the physiological effects responsible for such results. The present study aimed to evaluate the mechanisms modulated by dietary arginine supplementation to sows during lactation, regarding antioxidant capacity and vascularization of mammary glands. Lactating sows were fed control diet (CON) or CON diet supplemented with 1.0% L-arginine (ARG) for 21 days. ARG fed sows presented lower mRNA expression for *prolactin receptor* ($p = 0.002$), *angiopoietin1* ($p = 0.030$) and *receptor tyrosine kinase Tie2* ($p = 0.015$); higher expression for *prostaglandin synthase 1* ($p = 0.009$); trend of decrease for *glucocorticoid receptor* ($p = 0.062$) and *insulin-like growth factor receptor 1* ($p = 0.073$); and a tendency for an increased *glutathione peroxidase* ($p = 0.053$). The *angiopoietin2:angiopoietin1* mRNA ratio tended to increase ($p = 0.069$) in ARG fed animals. ARG fed sows demonstrated greater volumetric proportion of blood vessels ($p = 0.047$) and a trend of enhance in the number of blood vessels per mm^2 ($p = 0.073$). The outcomes pose that 1.0% L-arginine supplementation to sows activated proliferative mechanisms and enhanced antioxidant capacity at 21 days of lactation, besides promoting previous mammary tissues' angiogenesis.

RESUMO

HOLANDA, Débora Muratori, M.Sc., Universidade Federal de Viçosa, julho de 2017. **L-arginina melhora a vascularização e capacidade antioxidante da glândula mamária de porcas lactantes.** Orientador: Alysson Saraiva. Coorientadores: Márcio de Souza Duarte e Simone Eliza Facioni Guimarães.

As glândulas mamárias da porca lactante passam por períodos de incremento metabólico e estresse oxidativo. Estudos prévios demonstram que a suplementação de fêmeas suínas lactantes com arginina promove melhora no desempenho da leitegada, porém não abordam os efeitos fisiológicos responsáveis por tais resultados. O presente estudo teve como objetivo avaliar os mecanismos modulados pela suplementação dietética de arginina em porcas lactantes, quanto à capacidade antioxidante e à vascularização das glândulas mamárias. As porcas lactantes foram alimentadas com dieta de controle (CON) ou dieta CON suplementada com 1,0% de L-arginina (ARG) por 21 dias. As matrizes alimentadas com ARG apresentaram menor expressão de mRNA para *receptor de prolactina* (p = 0,002), *angiopoietina1* (p = 0,030) e *receptor tirosina quinase Tie2* (p = 0,015); maior expressão para *prostaglandina sintase 1* (p = 0,009); tendência de diminuição para *receptor de glicocorticóide* (p = 0,062) e *receptor do fator de crescimento semelhante à insulina 1* (p = 0,073); e tendência para aumento da *glutationa peroxidase* (p = 0,053). A proporção de mRNA de *angiopoietina1:angiopoietina2* tendeu a aumentar (p = 0,069) em animais alimentados com ARG. As porcas alimentadas com ARG demonstraram maior proporção volumétrica de vasos sanguíneos (p = 0,047) e uma tendência de aumento no número de vasos sanguíneos por mm^2 (p = 0,073). Os resultados sugerem que 1,0% de suplementação de L-arginina em porcas ativaram mecanismos proliferativos e propiciaram aumento da capacidade antioxidante aos 21 dias de lactação, além de promover, previamente, a angiogênese nos tecidos mamários.

L-arginine improves mammary gland vascularity and antioxidant capacity of lactating sows

D. M. Holanda[1]; C. S. Marcolla[2]; S. E. F. Guimarães[1]; M. M. Neves [3]; M. S. Duarte[1], M. L. T. Abreu[4]; A. Saraiva[1]

1 Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, Brazil.
2 Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada.
3 Department of General Biology, Universidade Federal de Viçosa, Viçosa, MG, Brazil.
4 Department of Animal Science, Universidade Federal de Lavras, Lavras, MG, Brazil.

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Abstract

Lactating sow's mammary glands pass through periods of metabolic burden and oxidative stress. The present study aimed to evaluate the mechanisms modulated by dietary arginine supplementation to sows during lactation, regarding antioxidant capacity and vascularization of mammary glands. Lactating sows were fed control diet (CON) or CON diet supplemented with 1.0% L-arginine (ARG) for 21 days. ARG fed sows presented lower mRNA expression for *prolactin receptor* (p = 0.002), *angiopoietin1* (p = 0.030) and *receptor tyrosine kinase Tie2* (p = 0.015); higher expression for *prostaglandin synthase 1* (p = 0.009); trend of decrease for *glucocorticoid receptor* (p = 0.062) and *insulin-like growth factor receptor 1* (p = 0.073); and a tendency for an increased *glutathione peroxidase* (p = 0.053). The *angiopoietin2:angiopoietin1* mRNA ratio tended to increase (p = 0.069) in ARG fed animals. ARG fed sows demonstrated greater volumetric proportion of blood vessels (p = 0.047) and a trend of enhance in the number of blood vessels per mm^2 (p = 0.073). The outcomes pose that 1.0% L-arginine supplementation to sows activated proliferative mechanisms and enhanced antioxidant capacity at 21 days of lactation, besides promoting previous mammary tissues' angiogenesis.

Keywords: functional amino acid, gene expression, histology, lactation, pig.

Introduction

Arginine and its byproducts fulfill numerous functions in the body, such as protein synthesis, ammonia detoxification, cell proliferation, hormone secretion, apoptosis regulation, immune response, tissue repair, blood flow and body's antioxidant capacity (Wu and Morris, 1998; Flynn et al., 2002; Kim et al., 2007; Wu et al., 2009). Sow's mammary complex must develop to undertake lactation (Farmer, 2013) and animals are subjected to metabolic burden during this phase (Berchieri-Ronchi et al., 2011), resulting in higher reactive oxygen species (ROS) production, especially of mitochondrial origin (Ott et al., 2007). The ROS cause oxidative stress when in imbalance with antioxidant factors (Valko et al., 2007), damaging organic constituents and culminating in cell death (Limón-Pacheco and Gonsebatt, 2009).

Arginine is a conditionally essential amino acid to animals under oxidative stress situations (Wu, 2009; Wu et al., 2009). Previous studies evaluating effects of dietary arginine supplementation to lactating sows have shown performance improvements for nursing piglets and no changes for lactating sows (Mateo et al., 2007; Lima, 2010; Cui et al., 2017). Piglet weight gain is related to milk composition and (Farmer et al., 2010) and arginine uptake by mammary glands is higher than its concentration on milk (Manjarin et al., 2014). Considering arginine functional roles, it is possible that the amino acid may have other functions in mammary tissues beyond milk protein constitution. For example, promoting cell antioxidative pathways, increasing blood flow and enhancing nutrient supply, justifying reported improvements on animal performance. However, the physiological responses promoted by arginine in lactating sows are undetermined. Therefore, the present work aimed to verify the mechanisms modulated by the dietary supplementation of 1.0% of L-arginine in lactating sows, evaluating mammary gland vascular function and antioxidant capacity.

Material and methods

Animals and housing

This study was approved by the animal ethics committee of the Animal Science Department, protocol 053/2015, and performed in the pig facility of the *Universidade Federal de Viçosa* (UFV), Brazil. Eleven commercial crossbred gestating sows and gilts were used. At 109 days of gestation, animals were transferred to individual farrowing crates equipped with manual feeders and automatic drinker bowls. Farrowing rooms were equipped with datalogger devices (Akso, São Leopoldo, Brazil) to register environmental

temperature and humidity every 15 minutes. Animals were fed gestation diet until farrowing.

Experimental design and diets

At farrowing, considered day zero (D0), sows were assigned in a completely randomized design into two treatments according to lactation diets. Control lactation diet (CON) was formulated to meet or exceed nutritional requirements of lactating sows, as recommended by Rostagno et al. (2011). Control group was composed by three animals of first and two of third parities, while three of first and three of third parities were on arginine supplemented group. Experimental diets were sent to *CBO Análises Laboratoriais* (Campinas, Brazil) to determine crude protein concentration by the Dumas method (MAPA, 1998) and amino acids' concentration by HPLC (White et al., 1986; Hagen et al., 1989), except for tryptophan, in which enzymatic analysis was performed (Lucas and Sotelo, 1980). The supplementation of 1.0% L-arginine on lactation diet (ARG) was done substituting clay filler for L-arginine (Ajinomoto, Saga, Japan). Nutritional levels for metabolizable energy (3,450 kcal/kg), calcium (0.830%), phosphorus (0.430%) and digestible amino acids, lysine (0.960%), methionine and cystine (0.524%), threonine (0.680%), tryptophan (0.214%) and valine (0.816%) were kept stable for both lactation diets. There were changes between CON and ARG diets for levels of crude protein (18.86 vs. 19.42%) and digestible arginine (1.250 vs. 2.030%).

The level of 1.0% of L-arginine supplementation in the ARG diet was chosen based on previous works and on its ability to increase arginine plasma concentration up to 100% in pigs (Cui et al., 2017). In order to avoid ammonia intoxication and competition for basic amino acid transporters, it was sought not to exceed 2.0% of arginine content in both diets, neither go beyond digestible arginine to digestible lysine ratio of 3.0, as recommended for sows (Wu et al., 2013). Hence, the concentration of 2.0% of digestible arginine and 2.1 ratio of arginine to lysine in ARG diet could not result in antagonism in intestinal absorption of lysine nor histidine.

Experimental diets were provided in individual feeders until D21. Every day, before the firs meal, feed wastage was weighed to calculate daily feed intake per animal. Sows were fed 6.0 kg/day, subdivided equally in four delivery times (07h00, 10h00, 13h00 and 16h00). Nursing piglets had no access to creep feed. Lactating sows and suckling piglets had free access to water throughout experimental period.

Data and sample collection

Sows were weighted with 109 days of gestation, 232.7 ± 37.4 kg mean weight, on D1 and at weaning (D21). On D0, D1 and D21 piglets were weighed individually. On D1, litters were cross-fostered to obtain 11 contemporary piglets per sow. On weaning, sows were exsanguinated after electro-stunning to obtain blood and tissue samples. Blood was collected, from sows' external pudendal vein using hypodermic needle (40 x 1.6, Injex, Ourinhos, Brazil) in siliconized tubes (PolyMed, Houston, USA), to obtain blood serum. Serum samples were stored at -20 °C for further hematological analysis. The time elapsed between weaning and slaughter was shorter than one hour.

Whole mammary complex was sectioned for weighting (Balmak, Santa Bárbara D'Oeste, Brazil), not taking skin load into account. Glandular units were weighted individually. Mammary gland tissue samples were obtained from the three cranial gland pairs, based on their greater uniformity in size and milk production among all glands (Kim et al., 2000 b). Fragments were sampled from the most central portion of mammary glands 4.0 cm caudal to teat nipples. For gene expression, samples of 0.25 cm^3 were disposed in tubes filled with RNAlater (Life Technologies, Carlsbad, EUA) and stored at 4.0 °C (adapted from Bustin and Nolan, 2004 a). For histological analysis, samples of 1.0 cm^3 were obtained from the same glandular region. Histological samples were fixed in 10% buffered formalin solution and stored at room temperature. Twenty-four hours after slaughter, gene expression samples were transferred to a new RNAlater solution and stored at -80 °C for further RNA extraction to gene expression analysis. Meanwhile, histological samples were transferred to a 70% ethanol solution for storage at room temperature.

Hematological analysis

Serum samples were sent to a clinical laboratory to quantify urea (Ureal Cobas c 311, Roche Diagnostics GmbH, Basel, Switzerland), IGF1 (Immulite® 2000 IGF-1 Kit, Siemens Healthineers, Erlangen, Germany) and insulin (Kit Architect Insulina, Abbott Laboratórios do Brasil Ltda., São Paulo, Brazil).

Gene expression analysis

Gene expression analysis of the first pair of mammary glands were performed at the *Laboratório de Biotecnologia Animal* (LABTEC) of the Animal Science Department of the UFV. Fifty milligrams of glandular tissues were used in RNA extraction procedures. Extraction was performed combining trizol (Life Technologies, Carlsbad,

EUA) and RNeasy Mini kit (Qiagen, Hilden, Germany) methods. cDNA synthesis was performed according to Goscript Reverse Transcription System protocol (Promega, Madison, EUA) in Veriti 96 Well thermocycler (Applied Biosystems, Foster City, EUA).

Primers' sequences for endothelial nitric oxide synthase (*ENOS*; Jayachandran et al., 2005), IGF receptor 1 (*IGF-1R*; Pierzchala et al., 2012), IGF binding protein 3 (IGFBP3; Wang et al., 2009) and glucocorticoid receptor (*GLU-R*; Poletto et al., 2006) were taken from previous studies. Other oligonucleotide sequences (Table 1) were designed with gene sequences obtained from GenBank database (https://www.ncbi.nlm.nih.gov/genbank) using PrimerQuest tool (http://www.idtdna.com/Primerquest/Home/Index). The primers had their specificity verified in silico, using Nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The optimization for each pair of primers was performed (Livak and Schmittgen, 2001). Primers were used for reverse transcription quantitative PCR (RT-qPCR) with GoTaq qPCR Master Mix (Promega, Madison, EUA) in ABI Prism 7300 Sequence Detection Systems thermocycler (Applied Biosystems, Foster City, EUA).

As candidates for housekeeping genes *β-actin*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) genes were tested. Among candidate endogenous control genes, *β-actin* and *GAPDH* were selected based on amplification and dissociation curves stability, efficiency of RT-qPCR and results from BestKeeper algorithm (Pfaffl et al., 2004). Geometric means of Ct values of *β-actin* and *GAPDH* were used to normalize target genes expression (Vandesompele et al., 2002). Gene of interest relative expression was calculated by: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Histological analysis

Samples from the first pair and third right glands were selected for histological analysis, based on representativeness of samples used for gene expression along with homogeneity of gland weights (data not shown) and visual conformation among all animals. The samples stored in 70% ethanol solution were cut into 1 mm-thick slices. The slices were dehydrated in crescent ethanol series (70, 80, 90 and 100%) and embedded in paraffin. Sections with a thickness of 5.0 μm were obtained using a rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany), stained with hematoxylin-eosin solution and qualitatively analyzed using light microscope (BX53, Olympus, Tokyo, Japan). For morphometric analysis, 12 digital images of each glandular unit (n = 3) of all animals were obtained randomly on cellSens Dimensions 1.6 software (Olympus, Tokyo,

Japan) using light microscope equipped with digital camera CMOS 1.3 MP BioCAM (Takachiho, Japan) and analyzed with ImageJ software (v.1.50i, National Institutes of Health, Bethesda, USA).

The representative number of histological fields per animal was determined for volumetry analysis according to Neves and Marques Jr. (2002) with modifications. Thirty-six histological fields per animal were established as a representative number for all histological variables evaluated. The total number of adipocytes and blood vessels were assessed by counting, and the area of each histological image was measured. The volumetric proportion of mammary gland tissue was obtained by counting 4.488 points projected onto 12 images per gland captured in histological slides, totalizing 13.464 points per animal. Coincident points were registered in glandular components (parenchyma, blood vessels, connective tissue, and adipose cells). The percent of points in each component was calculated using the formula: volumetric proportion (%) = [number of points in each component / (4.488)] x 100.

Statistical analysis

Data was analyzed in SAS 9.0 (SAS Inst. Inc., Cary, USA) considering each sow as an experimental unit. Differences were considered at $p < 0.05$. P-values between 0,05 and 0,10 were considered a trend.

Gene expression data was analyzed using %QPCR_MIXED macro (www.msu.edu/~steibelj/JP_files/QPCR.html), according to methods proposed by Steibel et al. (2009). BestKeeper was used to obtain the Pearson correlation coefficient between target genes (Pfaffl et al., 2004).

The angiopoietin2 (*ANG2*) to angiopoietin1 (*ANG1*) ratio (*ANG2*:*ANG1*) was calculated based on relative expression of each gene ($2^{-\Delta Ct}$). *ANG2*:*ANG1* ratio was submitted to the Wilcoxon test, PROC NPAR1WAY (Sheskin, 2000; Pfaffl, 2004), in behalf of data were classified as non-parametric after the Shapiro-Wilk test ($p = 0.001$) through PROC UNIVARIATE.

For histological variables, each slide mean value was considered one repetition (three repetitions per experimental treatment). Analysis of variance (PROC ANOVA) was performed for data with normal distribution (Shapiro-Wilk: $p \geq 0.050$), number of blood vessels per mm^2 and percentage of blood vessels. Other histological variables were submitted to logarithmic transformation for analysis of variance. Number of adipocytes per mm^2 (Shapiro-Wilk: $p < 0.001$) and percentages of adipocytes (Shapiro-Wilk: $p < 0.001$) and parenchyma (Shapiro-Wilk: $p < 0.001$) did not present normal distribution

after logarithmic transformation, their raw data were evaluated under the Wilcoxon test, PROC NPAR1WAY.

Results

Mean values of room minimum and maximum temperatures were 19.57 ± 1.79 °C and 28.15 ± 1.46 °C, respectively; for relative humidity values obtained were 44.73 ± 13.45% of minimum and 72.70 ± 7.75% of maximum means. Based on thermoneutral range for lactating sows (12 to 22 °C), as suggested by Black et al. (1993), it can be inferred that animals in present study were submitted to periods of heat stress. Feed intake was similar ($p = 0.963$) between animals receiving CON or ARG diets, while arginine intake was higher ($p < 0.001$) in animals that received ARG diet. Sows' weights did not vary in pre-partum, post-partum or at slaughter (Table 2). The inclusion of arginine in lactation diet did not result in difference for hematological variables of IGF1 ($p = 0.960$), insulin ($p = 0.483$) or urea ($p = 0.161$) for sows on D21 (Table 3).

By the end of the third lactation week, histological analysis of mammary glands showed no difference between groups for the number of adipocytes per mm^2 ($p = 0.275$). However, a greater percentage of blood vessels ($p = 0.047$) and a trend of enhance on the number of blood vessels per mm^2 ($p = 0.073$) were found in animals treated with arginine than the controls. The percentage of other histological components of the mammary gland, such as adipocytes ($p = 0.682$), connective tissue ($p = 0.962$) and parenchyma ($p = 0.445$) did not differ between ARG and CON fed sows (Table 4).

No difference was observed for *ENOS* gene expression ($p = 0.708$) for sows fed ARG or CON diets. Prolactin receptor (*PRL-R*) presented lower mRNA expression ($p = 0.002$) in ARG fed sows. Equivalent mRNA expressions were observed among ARG and CON groups for *IGF1* ($p = 0.853$) as well as IGF binding protein 2 (*IGFBP2*, $p = 0.532$), *IGFBP3* ($p = 0.309$) and IGF binding protein 5 (*IGFBP5*, $p = 0.145$). Nevertheless, there was a trend of 1.92-fold decrease in expression for *IGF1-R* ($p = 0.073$) in sows fed ARG (Figure 1). The *GLU-R* presented a trend of lower ($p = 0.062$) mRNA expression in ARG fed animals. Prostaglandin synthase 1 (*COX1*) showed opposite behavior for sows given ARG, with increase ($p = 0.009$) in mRNA expression. Regarding antioxidant enzymes, greater mRNA expression was found for glutathione peroxidase (*GSH-PX*, $p = 0.053$) in ARG fed sows. For other antioxidant enzymes evaluated, there was no difference for catalase (*CAT*, $p = 0.270$), superoxide dismutase 1 (*SOD1*, $p = 0.566$) or superoxide dismutase 2 (*SOD2*, $p = 0.470$) for mRNA expression between experimental groups. No changes were observed for mRNA expression for lactoferrin (*LTF*, $p = 0.830$) among

treatments (Figure 2). Considering the mRNA expression of angiopoietins and receptor tyrosine kinase Tie2 (*TIE2*), there was reduction in *ANG1* ($p = 0.030$) and *TIE2* ($p = 0.015$) expressions in sows receiving ARG diet. The mRNA expression of *ANG2* did not differ ($p = 0.196$) between groups evaluated (Figure 3). The *ANG2*:*ANG1* mRNA ratio tended to increase ($p = 0.069$) in ARG fed sows (Figure 4). No changes were observed in mRNA expression for vascular endothelial growth factor (*VEGF*, $p = 0.401$), vascular endothelial growth factor receptor 1 (*VEGFR1*, $p = 0.446$) and vascular endothelial growth factor receptor 2 (*VEGFR2*, $p = 0.562$) (Figure 3). The Pearson's correlation coefficients were observed for target genes that presented differences in relative expression (Figure 5). There was correlation between *PRL*-R/COX1 ($r = -0.566$, $P = 0.006$), *PRL-R*/*ANG1* ($r = 0.554$, $p = 0.007$), *PRL-R*/*TIE2* ($r = 0.577$, $p = 0.005$), *IGF1-R*/*CAT* ($r = -0.621$, $p = 0.002$), *IGF1-R*/*COX1* ($r = -0.440$, $p = 0.041$), *IGF1-R*/*ANG1* $r = 0.453$, $p = 0.034$) and *IGF1-R*/*TIE2* ($r = 0.694$, $p = 0.001$).

Discussion

Heat stress, farrowing stress and the use of primiparous sows, which present low feed consumption, are among factors that may have contributed to the pre-determined daily feed supply of 6.0 kg of feed/day has not been reached. As feed intake did not vary among experimental groups, it can be stated that the highest intake of arginine observed occurred due to its distinct concentrations in experimental diets.

Increased milk protein content (Mateo et al., 2008) or milk production (Farmer et al., 2010), are related to heavier piglets by the end of the third lactation week. Thus, it is possible that arginine supplementation was capable to enhance nutrient supply, uptake or utilization in lactating glands.

Similar concentrations for serum urea found in this study indicate no excessive nitrogen supply in view of the supplementation with dietary arginine (Coma et al., 1995). Additionally, this outcome indicates no difference in nitrogen utilization efficiency among diets, coherent result with arginine:lysine ratio of 2.1, lower than recommended by Wu et al. (2013). Nielsen et al. (2001 b) and Mateo et al. (2008) reported that arginine requirement for lactating sows and gilts was higher in early lactation period, when breast tissues are rapidly growing to meet daily increase in milk production, supporting the present equivalent urea concentrations on D21. On the contrary, a recent study showed lower plasma urea levels for sows provided dietary arginine at 14 and 21 days of lactation (Cui et al., 2017). It seems that hematological urea levels present variable behavior, probably due to other experimental factors that still need to be clarified.

Our results demonstrate for the first time that 1.0% arginine supplementation to lactating sows incurs in highest number per mm^2 and percentage of blood vessels, suggesting increment in both counting and diameter of blood vessels (Figure 6). Polyamines and NO stimulate proliferation of endothelial cells (Li et al., 2001), indicating that arginine played its angiogenic role in mammary tissue in present study through these byproducts. Possibly bringing metabolic advantages to mammary glands, for example, greater nutrient yield caused by enhanced blood flow to the organ. Higher tissue vascularization, as a consequence of L-arginine supplementation, was also found in placental membranes of gestating gilts (Li et al., 2010), possibly due to increases *ENOS* gene expression (Wu et al., 2012). Then, the equivalent gene expression for *ENOS* in ARG and CON fed animals was unexpected, particularly after stating increased vascularity in mammary glands. This enzyme isoform has its activation modulated by interaction with calmodulin (Abu-Soud and Stuehr, 1993), consequently *ENOS* mRNA abundance is secondary to predict its metabolites production (Alderton et al., 2001). Additionally, *ENOS* higher levels could probably have been found during first days of arginine supplementation, when tissues are actively proliferating (Nielsen et al., 2001), justifying similar mRNA expressions for *ENOS* at weaning.

The relationship between both *ANG1* and *ANG2* is of great importance to estimate *TIE2* activation since angiopoietins compete for the same receptor (Hansen et al., 2010). The reduction for *ANG1* and *TIE2* mRNA expressions in addition to *ANG2:ANG1* 5.13 unit-increase in ARG fed sows, admits possible destabilization and restructuration of vascular mammary gland network by L-arginine supplementation. It can be inferred because increased *ANG2* expression can be observed at tissue remodeling sites (Maisonpierre, 1997) and *ANG1* alone does not induce endothelial cell multiplication (Davis et al., 1996). Instead, *ANG1* promotes intercellular adhesion and reduction of vascular permeability, both effects related to blood vessel stabilization (Stratmann et al., 1998; Kim et al., 2000 a). The *VEGF* related genes are indispensable to activate angiogenic pathways, including those mediated by angiopoietins (Eklund and Olsen, 2006). Then, the absence of difference in expressions for *VEGF*'s group reveals that vascular multiplication and modeling was probably diminished at sampling day (D21).

Arginine causes increases in circulating levels of prolactin, IGF and insulin in sows (Mateo et al., 2008; Cui et al., 2017), important hormones in mammary gland tissues' proliferation, differentiation (Fendrick et al., 1998) and nutrient uptake (Farmer et al., 2008). Increased mammary tissues sensitivity to insulin due to lactation might lead to unnecessary rise on this hormone concentration to provide greater nutrient uptake

(Burnol et al., 1990). Consistent with this view and corroborating the equivalent serum insulin concentrations among sows fed ARG and CON diets on D21, no insulin variation was observed at 21 days of lactation in sows receiving 1.73 versus 1.34 or 0.96% of arginine in diet (Laspiur et al., 2006). In contrast, elevated insulin concentration was found in sows fed 1.64 versus 0.82% of arginine content (Cui et al., 2017).

Indirect effect of GH on mammary tissues occurs through IGFs and IGFBPs (Allan et al., 2002), resulting in higher milk production, even though with lower participation than prolactin (Travers et al., 1996; Wilde et al., 1999). Contradictorily to possible higher milk output, due to acinar cells' proliferation, alike *IGF1* and *IGFBP*s expressions and IGF1 hematological concentrations were observed. Similar results were verified by Laspiur et al. (2006), finding no differences in plasma for GH, insulin or glucose at 21 days of lactation in sows supplemented with dietary arginine. The effect of *IGF*'s superfamily on mammary gland development may be more related to proliferation of mammary duct cells (Richert and Wood, 1999), which is more significative during postnatal mammary gland development (Kensinger et al., 1982). Hence, *IGF1* gene expression shall be more relevant at the beginning of glandular development period in comparison to the end of lactation. Moreover, *IGFBP*s present great variability in gene expression levels as well as in their location in the mammary gland of mice females (Wood et al., 2000). Supposing analogous observations in sows, it would be compatible with *IGFBP*s mRNA expression patterns found in present study. Furthermore, it is possible that both arginine levels used in current study (1.25 and 2.03%) were higher enough to promote arginine secretagogue effect on insulin and IGF1, indeed no difference was found for CON and ARG fed sows.

The observed decrease in *PRL-R* gene expression reinforces previous findings, indicating elevation of circulating prolactin (Farmer and Palin, 2005), in response to arginine supplementation during lactation (Cui et al., 2017), counteracting the hormone's behavior to decrease its level during lactation (Farmer et al., 2010). Of note, prolactin has greater importance than IGF's group on milk synthesis and prevention of acinar cell apoptosis in rat mammary glands (Travers et al., 1996), being responsible for higher milk yield (VanKlompenberg et al., 2013). Such information sustains the existence of alternative pathways other than those modulated by GH and its mediators for cell survival and metabolism on mammary glands. Among possible effects of arginine at cellular level is the activation of proliferative and anabolic mechanisms mediated by NO (Du et al., 1997), mTOR (Tan et al., 2010; Kim et al., 2013) and polyamines (Wu et al., 2009). The

Pearson's correlation coefficient for *PRL-R*/*ANG1* and *PRL-R*/*TIE2* highlight interactions between proliferative and angiogenic pathways that we could not explain.

Another effect of L-arginine supplementation was the reduction of 2.39-fold for *GLU-R*. The expression of *GLU-R* is elevated in the beginning of lactation (Manjarín et al., 2012). Glucocorticoids act as lactogenic hormones (Kobayashi et al., 2017) and on cell differentiation of mammary alveolar epithelium (Casey and Plaut, 2007), potentiating protein synthesis stimulated by prolactin for maintenance of lactation (Doppler et al., 2001, 2002; Manjarín et al., 2012). Despite these effects of glucocorticoids, elevated *GLU-R* expression would be undesirable to observe responses to arginine supplementation. Glucocorticoids increase metabolic rate of arginine in intestinal piglet cells (Flynn and Wu, 1997; Wu et al., 2000 a; b) and hinder the amino acid transport and decrease its availability in tissues (Shi et al., 2004), leading to possible consequent restrain of tetrahydrobiopterin (BH4) and NO synthesis and compromising arginine metabolites regulatory functions. To avoid inconsistent supply of arginine to organs, the amino acid is capable to reduce *GLU-R* expression and decrease circulating cortisol level in finishing pigs (Ma et al., 2010). Additionally, the secretagogue effect of arginine on prolactin, suggested by the reduction in the expression of its *PRL-R*, may have contributed to the decrease in the expression of the *GLU-R*, considering that both hormone receptors are related to stimulation of milk protein synthesis and the increment in one's hormone concentration turns unnecessary the other's presence to ensure lactopoiesis (Kobayashi et al., 2017).

There is a lack of studies regarding COX1 roles in normal mammary glands, especially during lactation. Nevertheless, it has been shown that *COX1* transcripts are more abundant during the initial lactation period compared to other phases of rodents' reproductive cycle, presenting progressive reduction on its expression during lactation (Chandrasekharan et al., 2005). The higher relative gene expression for *COX1* by 1.85-fold indicates greater synthesis of prostanoid metabolites (Badawi and Archer, 1998; Chandrasekharan et al., 2005), stimulating a cascade of intracellular events, through increased calcium influx (revised by Bos et al., 2004) and promoting cell differentiation (Chandrasekharan et al., 2005), proliferation (Bandyopadhyay et al., 1987), angiogenesis (Majima et al., 2003) and antiapoptotic mechanisms (Sheng et al., 1998). Thus, the elevation of *COX1* expression, provided by L-arginine supplementation, may prolong growth and differentiation periods of constitutive mammary gland tissues. The negative correlation coefficient observed between *PRL-R*/*COX1* expressions proposes *COX1* expression and can be modulated by prolactin level, indicating that hormonal proliferative

effects of prolactin may be mediated by COX1 activity. Likewise, increased intracellular calcium influx may potentiate NO-mediated arginine responses, considering *ENOS* activation dependence on calmodulin interaction (Abu-Soud and Stuehr, 1993). Despite the results found on previous works, more studies are required to characterize COX1 roles during lactation period, particularly in sows.

The increase of 2.04-fold in transcript expression for *GSH-PX*, important intracellular antioxidant enzyme, was observed in ARG fed animals. Comparable outcome was observed by supplementation with dietary arginine for finishing animals (Ma et al., 2010), indicating that higher arginine intake can increase antioxidant mechanisms. No difference for other antioxidant enzymes evaluated demonstrates no significant increase or reduction in oxidative stress at D21 to cause alteration on such enzymes.

The *IGF-1R* is related to increased resistance to oxidative stress through challenging situations in rat cell culture (Chen et al., 2014). The decrease of 1.92-fold in expression of *IGF1-R* in sows fed ARG might indicate augments in antioxidant capacity of mammary glands, by the increase in antioxidant factors, such as the elevation of 2.04-fold in *GSH-PX*, or lower activation of pro-oxidant mechanisms modulated by arginine. Arginine provision allows a reduction in ROS synthesis enhancing the bioavailability of BH4 and NO. The BH4 is cofactor of ENOS (Shi et al., 2004) and its deficiency increases superoxide production (Stroes et al., 1998), while NO is capable interact and inactivate ROS (Gardner et al., 2001). Deficient vascularity may lead to oxidative stress and stimulates angiogenic pathways (Forsythe et al., 1996). The IGF1-R lesser expression indicates low oxidative stress, turning angiogenesis dismissible during late lactation, as observed through the positive Pearson's correlation coefficients found for *IGF1-R*/*ANG1* and *IGF1-R*/*TIE2*. The *IGF1-R*/*COX1* negative correlation reveals that proliferative mechanisms mediated by IGFs was possibly replaced by *COX1* responses.

In conclusion, L-arginine supplementation to lactating sows promoted angiogenesis, vasodilation and activation of proliferative mechanisms, besides enhance in antioxidant capacity. Animal nutritional efficiency, due to lower mammary gland tissues damage and subsequent repair rates in addition to increased blood flow and activation of anabolic pathways, supports the higher piglet weaning weight of sows supplemented with the amino acid described in previous works.

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Appendix

Table 1 *Oligonucleotides used on gene expression analysis*

Gene	GenBank no.	Sequence
Angiogenesis		
ENOS	NM_214295.1	F: CAAAGTGACCATTGTGGACCAT R: TGCTCGTTCTCCAGGTGCTT
ANG1	NM_213959.1	F: ACAGAGCCACCACCAATAAC R: GTGCAAAGGTTGACGAGATTATG
ANG2	NM_213808.1	F: CTGAGCTGTGATCTCGTCTTG R: CTGAACCTGATACTGCCTCTTC
TIE2	XM_001926034.5	F: CGGCACGAAGTACCTGATATT R: GGTGGAAGAGGTTTCCTCCTATG
VEGF	NM_214084.1	F: GCACATAGGAGAGATGAGCTTC R: CAAGGCCCACAGGGATTT
VEGFR-1	EU714325.1	F: AGACTCGGGCACCTATG R: GGCTTGCCGATCTCTAATG
VEGFR-2	NM_214084.1	F: GATGCTCGCCTCCCTTTGA R: AGTTCCTTCTTTCAGTCGCCTACA
Proliferation		
IGF1	NM_214256.1	F: CGCTCTTCAGTTCGTGTG R: GAAGCAGCACTCATCCAC
IGF-1R	NM_214172.1	F: TGGATGCCGTGTCCAATAACT R: ACAAGTCCCCGCATTCCTT
IGFBP2	NM_214003.1	F: AGCATGGCCTGTACAACCTCAAAC R: CTGCTGCTCGTTGTAGAAGAGAT
IGFBP3	AY422045	F: GTCCACACCAAGATGGACGTGAT R: CATGTTCAGGAACTTGAGGTGGT
IGFBP5	NM_214099.1	F: AGCCAAGATCGAGAGAGAC R: TGCTTGGGTCGGAAGAT
PRL-R	NM_001001868.1	F: CTCCTGTGAACCTGACTTTG R: GTGAGCCAACCAGATCTTAC
GLU-R	AF141371	F: GATCATGACCGCACTCAACATG R: TTGCCTTTGCCCATTTCAC
COX1	XM_001926129.5	F: GGGTCTCTTATCCGTGATCT R: GAGCCCAGTGTTCATTCTG
Antioxidative capacity		
LTF	M81327.1	F: GTGCATAGGGAAAGGGAAAG R: GTTGAAGGCACCGGAATAG
CAT	D89812.1	F: CTGCATCAGGTTTCCTTCC R: CGCCTCTCCCTTCTCATTA

Table 1 *Continued*

Gene	GenBank no.	Sequence
GSH-PX	AF532927.1	F: CGTCGCTTTCTGACCATC R: CCCGAGAGTAGCACTGTAA
SOD1	GU944822.1	F: GGCAGAGGTGGAAATGAAG R: CCATGGCATGAGGGAATG
SOD2	NM_214127.2	F: GACCTGCCGTACGACTAT R: GTTCAGGTTGTTCACGTAGG
House-keeping		
β-actin	U07786.1	F: CTCTTCCATCGTGTCCTTCTAC R: CCTCAGACTTGTCGATCTTCTG
GAPDH	NM_001206359.1	F: CAAAGTGGACATTGTCGCCATCA R: AGCTTCCCATTCTCAGCCTTGACT
HPRT1	NW_003540069.1	F: GCTGACCTGCTGGATTACAT R: CTGGTCATTACAGTAGCTCTTCAG

ENOS, endothelial nitric oxide synthase; *ANG1*, *angiopoietin1; ANG*2, angiopoietin2; *TIE2*, receptor tyrosine kinase Tie2; *VEGF*, vascular endothelial growth factor; *VEGFR-1*, vascular endothelial growth factor receptor 1; *VEGFR-2*, vascular endothelial growth factor receptor 2; *IGF-1R*, IGF receptor 1; *IGFBP2*, IGF binding protein 2; *IGFBP3*, IGF binding protein 3; *IGFBP5*, IGF binding protein 5; *LTF*, lactoferrin; *PRL-R*, prolactin receptor; *GLU-R*, glucocorticoid receptor; *COX1*, prostaglandin synthase 1; *CAT*, catalase; *GSH-PX*, glutathione peroxidase; *SOD1*, superoxide dismutase 1; *SOD2*, superoxide dismutase 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, hypoxanthine-guanine phosphoribosyltransferase.

Table 2 *Sows' performance variables during a 21-day lactation period*

Items	CON	ARG	SD	p-Value
Feed intake, kg/day	5.259	5.241	0.60	0.963
Digestible arginine intake, g/day	65.7	106.4	23.74	<0.001
Pre-partum weight, kg	230	235	37.34	0.827
Post-partum weight, kg	210	219	29.43	0.654
Slaughter weight, kg	204	207	27.23	0.860

SD, mean standard deviation.

Table 3 *Serological analysis of IGF1, insulin and urea of sows at 21 days of lactation*

Variables	CON	ARG	SD	p-Value
IGF1, ng/mL	103.40	104.83	44.21	0.960
Insulin, mcUI/mL	7.94	6.20	3.73	0.484
Urea, mg/mL	38.40	44.17	5.92	0.161

SD, mean standard deviation.

Table 4 *Histomorphometric variables of sows' mammary glands at 21 days of lactation*

Variable	CON	ARG	SD	p-Value
Number, /mm^2				
Adipocytes	17.78	18.28	32.72	0.275
Blood vessels	22.90	30.09	10.92	0.073
Volumetric proportion, %				
Adipocytes	1.18	1.23	2.36	0.682
Blood vessels	0.95	1.49	0.78	0.047
Connective tissue	7.76	7.87	3.53	0.962
Parenchymal tissue	90.11	89.41	5.18	0.445

SD, mean standard deviation.

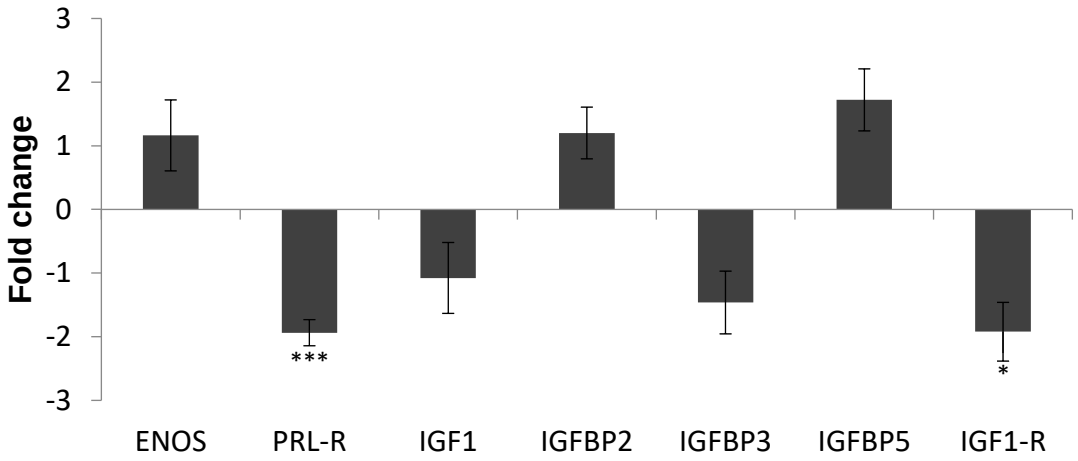

Figure 1 Fold change between 1.0% dietary L-arginine supplementation (ARG) and control diet (CON) on the relative expression (positive values mean higher gene expression for ARG fed sows) of endothelial nitric oxide synthase (*ENOS*) and respective genes related to cell proliferation in sows' mammary gland at 21 days of lactation. *PRL-R*, prolactin receptor; *IGFBP2*, IGF binding protein 2; *IGFBP3*, IGF binding protein 3; *IGFBP5*, IGF binding protein 5; *IGF-1R*, IGF receptor 1. *** $p < 0.01$; * $0.05 < p < 0.10$.

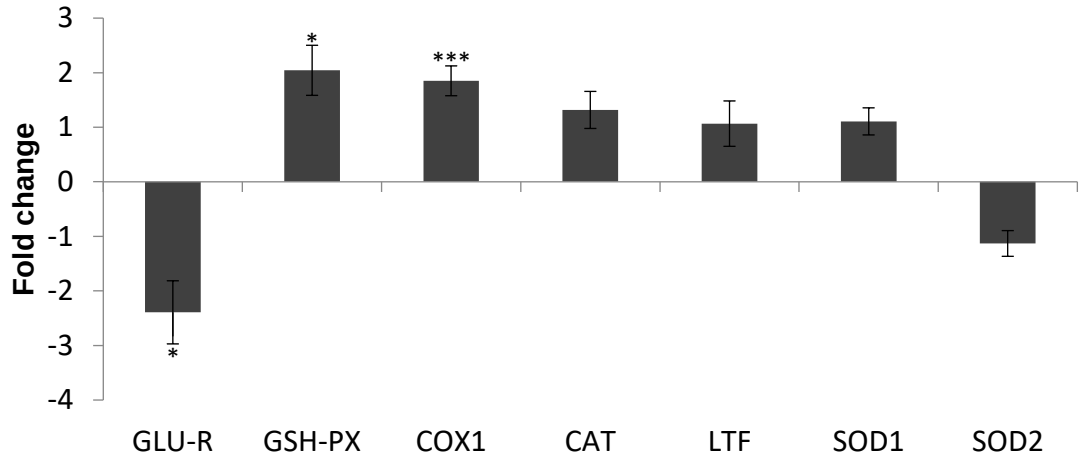

Figure 2 Fold change between treatments with 1.0% L-arginine supplementation (ARG) and control (CON) diets relative to the relative expression (positive values mean higher gene expression for ARG fed sows) of the respective genes related to antioxidant capacity in sows' mammary glands at 21 days of lactation. *GLU-R*, glucocorticoid receptor; *GSH-PX*, glutathione peroxidase; *COX1*, prostaglandin synthase 1; *CAT*, catalase; *LTF*, lactoferrin; *SOD1*, superoxide dismutase 1; *SOD2*, superoxide dismutase 2. *** $p < 0.01$; * $0.05 < p < 0.10$.

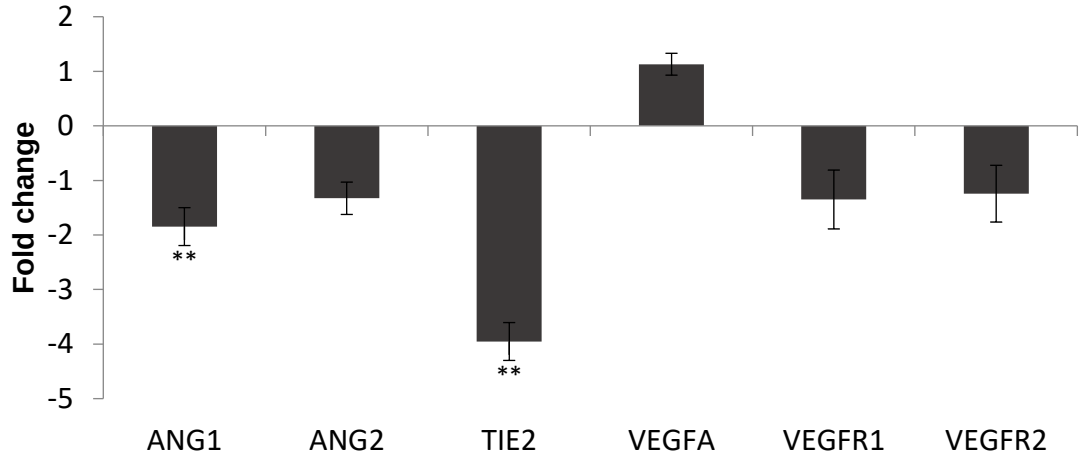

Figure 3 Fold change between treatments with 1.0% L-arginine supplementation (ARG) and control (CON) diets regarding relative expression (positive values mean higher gene expression for ARG fed sows) of respective genes related to angiogenesis in sows' mammary glands at 21 days of lactation. *ANG1*, angiopoietin 1; *ANG2*, angiopoietin 1; *TIE2*, receptor tyrosine kinase Tie2; *VEGFA*, vascular endothelial growth factor A; *VEGFR1*, vascular endothelial growth factor receptor 1; *VEGFR2*, vascular endothelial growth factor receptor 2. ** $0.01 < p < 0.05$.

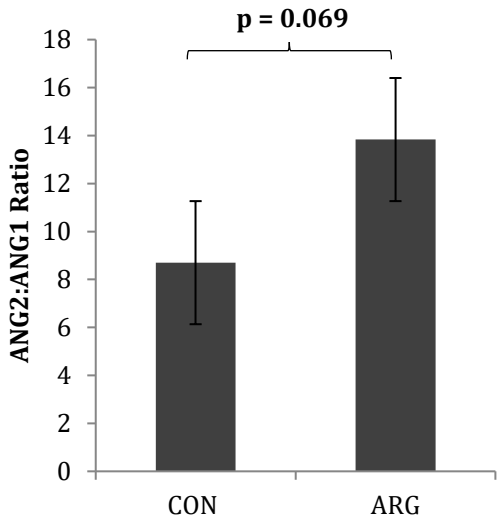

Figure 4 Ratio between relative gene expression of angiopoietin2 (*ANG2*) and angiopoietin1 (*ANG1*) for control (CON) and supplemented with 1.0% L-arginine (ARG) sows at 21 days of lactation.

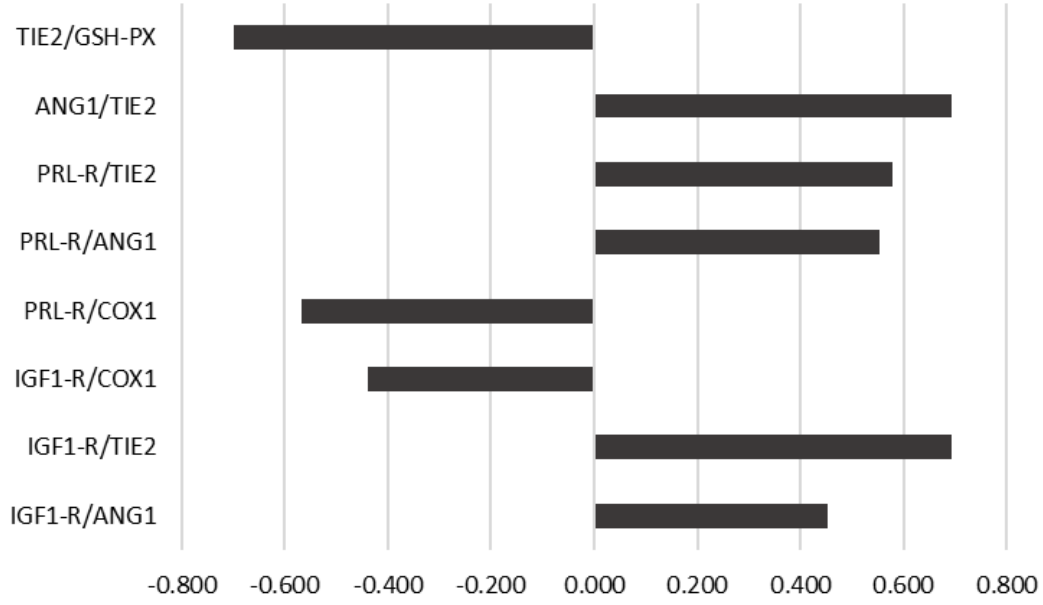

Figure 5 The Pearson's correlation coefficient between differently expressed target genes for sows at 21 days of lactation. *TIE2*, receptor tyrosine kinase Tie2; *GSH-PX*, glutathione peroxidase; *ANG1*, angiopoietin1; *PRL-R*, prolactin receptor; *COX1*, prostaglandin synthase 1; *IGF-1R*, IGF receptor 1.

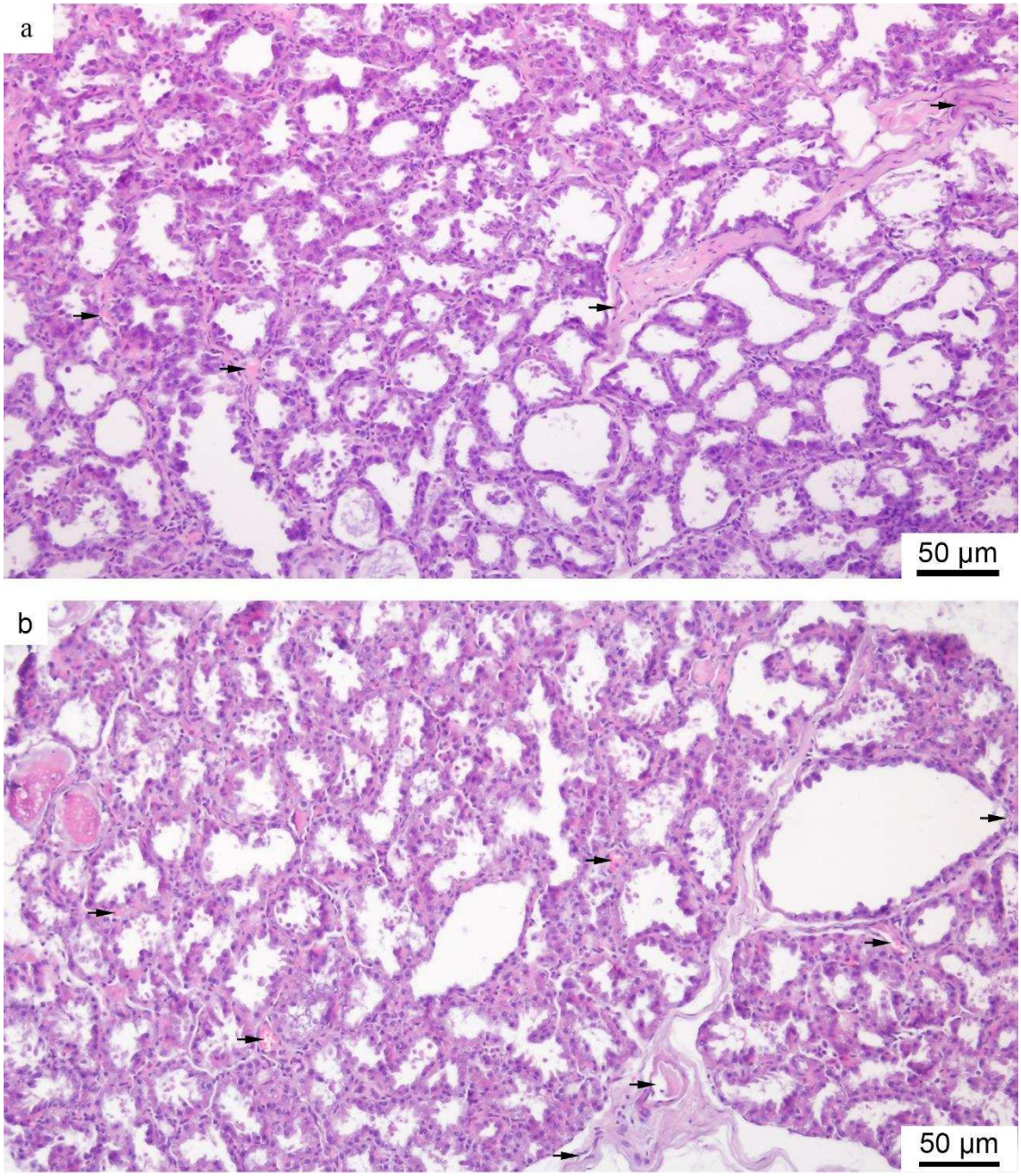

Figure 6 Light micrographs of histological sections of lactating sows' mammary glands from animals receiving control diet (a) or 1.0% L-arginine supplemented diet (b). Arrows indicate blood vessels.

Annex

UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
CEUAP/UFV

Campus Universitário – Viçosa, MG – 36570-900 – Telefone:(31) 3899.3275 – e-mail: ceuap@ufv.br – site: www.ceuap.ufv.br

Viçosa, 18/09/15

CERTIFICADO

Certificamos que o projeto intitulado **"Arginina e valina na nutrição de matrizes suínas hiperprolíficas"**, protocolo **nº 053/2015**, sob a responsabilidade de **Alysson Saraiva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo conselho nacional de controle da experimentação animal (concea), e foi aprovado pela comissão de ética no uso de animais de produção da universidade federal de viçosa (ceuap-ufv) em reunião de **18/Set/2015.**
Vigência do Projeto: de **01/10/2015** a **31/12/2015**
Espécie/linhagem: **Suínos** Nº de animais: **20**
Peso: **270Kg** Idade: **18 meses** Sexo: **Fêmea** Origem: **Setor de Suínocultura**

CERTIFICATE

We certify that the project entitled **"Arginine and valine in the nutrition of hyper-prolific sows"** protocol **nº 053/2015**, under the responsibility of **Alysson Saraiva** - which involves the production, maintenance and / or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **Sep, 18th, 2015**.
Duration of the Project: from **Oct, 01st, 2015 to Dec, 31th, 2015**.
Species / strain: **Swine** Nº of animals: **20**
Weight: **270Kg** Age: **18 months** Sex: **Female** Source: **Setor de Suínocultura**

Mário Luiz Chizzotti
Coordenador da CEUAP/UFV