

SÃO PAULO STATE UNIVERSITY  
INSTITUTE OF BIOSCIENCES  
POSTGRADUATE PROGRAM IN PHARMACOLOGY AND BIO-  
TECHNOLOGY

**Study of genes stimulated by interferon- $\tau$  in immune  
cells as candidates for pregnancy markers in bovine fe-  
males**

**ISABELLA RIO FELTRIN**

Botucatu – SP  
2024

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**ISABELLA RIO FELTRIN**

Thesis submitted to the Postgraduate Program in  
Pharmacology and Biotechnology of the Institute  
of Biosciences at the São Paulo State University to  
obtain the PhD degree in Pharmacology and Bio-  
technology.

**Advisor:** Claudia Maria Bertan Membrive, PhD.

**Co-Advisor:** Guilherme Pugliesi, PhD.

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## CERTIFICADO

Certificamos que a proposta intitulada "Inovações no diagnóstico precoce da gestação em bovinos", protocolada sob o CEUA nº 8192280317, sob a responsabilidade de **Guilherme Pugliesi** - 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 - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 10/05/2017.

We certify that the proposal "Innovations in the early pregnancy diagnosis in cattle", utilizing 90 Bovines (90 females), protocol number CEUA 8192280317, under the responsibility of **Guilherme Pugliesi** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 05/10/2017.

Finalidade da Proposta: **Pesquisa**

Vigência da Proposta: de **05/2017 a 04/2018** Área: **Reprodução Animal**

Origem:	Prefeitura do Campus da USP de Pirassununga		
Espécie:	Bovinos	sexo:	Fêmeas
Linhagem:	Nelore	idade:	3 a 9 anos
		Peso:	400 a 700 kg
		N:	30
Origem:	Prefeitura do Campus da USP de Pirassununga		
Espécie:	Bovinos	sexo:	Fêmeas
Linhagem:	Nelore	idade:	12 a 24 anos
		Peso:	300 a 400 kg
		N:	30
Origem:	Prefeitura do Campus da USP de Pirassununga		
Espécie:	Bovinos	sexo:	Fêmeas
Linhagem:	Holandesa	idade:	2 a 7 anos
		Peso:	450 a 700 kg
		N:	30

Resumo: A identificação do estado gestacional mais precocemente aos métodos atuais realizados por volta do 30º dia pós-inseminação permite uma redução no intervalo de inseminações, o que pode resultar em menor período para concepção e maior rentabilidade ao sistema de produção pecuário. A tese central do projeto é que biomarcadores no sangue estimulados pelo conceito são detectáveis no início da gestação. Isto pode permitir o desenvolvimento de um método precoce e inovador de diagnóstico da gestação em bovinos. Assim, tem-se como objetivo principal desenvolver um método de diagnóstico através da quantificação da abundância de genes estimulados por IFN-tau (ISGs) já conhecidos ou por novos marcadores aliado a uma identificação precoce dos animais não-gestantes pela avaliação da função luteal. Para isso, no Estudo 1 novos marcadores do estado gestacional poderão ser identificados através do estudo transcriptômico das células imunes do sangue no dia 14 e 18 pós-inseminação. Pretende-se caracterizar a abundância de ISGs já conhecidos e de novos transcritos estimulados pela presença do conceito, no sangue total, nas células imunes polimorfonucleares (PMNs) e em células imunes do leite entre os dias 12 e 20 de gestação em vacas de corte e leite. A luteólise será detectada por ultrassonografia Doppler através das mudanças no tamanho e vascularização do corpo lúteo (CL) e pelas concentrações circulantes de progesterona (P4) para detecção de vacas com CL não-funcional. Para mensuração das concentrações de P4 objetiva-se desenvolver e validar um ensaio imunoenzimático com alta sensibilidade e especificidade para evitar o uso de radioisótopos. No Estudo 2 será determinado a acurácia de métodos de diagnóstico da gestação realizados antes do dia 20 pós-IA, e baseados no uso isolado ou combinado da expressão de ISGs e da funcionalidade luteal (pela concentração de P4 ou ultrassonografia Doppler). Os novos conhecimentos e métodos inovadores aqui propostos embasarão o desenvolvimento de tecnologias para a pecuária de leite e corte. A exequibilidade será garantida pelo trabalho integrado de especialistas em cada etapa analítica e pela infraestrutura das instituições envolvidas.

Local do experimento: **CBRA/VRA/FMVZ**

São Paulo, 12 de maio de 2017



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*I dedicate this thesis to my parents, Ana and Feltrin, who  
are the basis of my life and my greatest supporters. Everything I  
am today is thanks to you!  
All my love for you!*

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## RESUMO

FELTRIN, I.R. **Estudo de genes estimulados por interferon- $\tau$  em células imunes como candidatos à marcadores de prenhez em fêmeas bovinas.** [Study of genes stimulated by interferon- $\tau$  in immune cells as candidates for pregnancy markers in bovine females]. 2024. 99 f. Tese (Doutorado em Farmacologia e Biotecnologia) – Instituto de Biociências, Universidade Estadual Paulista, Botucatu, 2024.

Nas fêmeas bovinas, as perdas embrionárias ocorridas entre o 14º e o 19º dia após o estro afetam significativamente a eficiência reprodutiva dos rebanhos. Portanto, o desenvolvimento de métodos diagnósticos capazes de detectar precocemente a gestação ou não gestação é um ponto importante para melhorar o desempenho reprodutivo dos bovinos. O interferon- $\tau$  (IFN- $\tau$ ) produzido pelo concepto desempenha um papel importante durante o reconhecimento materno da prenhez (MPR) em ruminantes, que envolve mais do que a inibição dos pulsos luteolíticos da prostaglandina F2 alfa (PGF<sub>2 $\alpha$</sub> ) para manter a funcionalidade do corpo lúteo (CL). Para uma prenhez bem-sucedida, o concepto semi-alogênico deve evitar a sua rejeição pelo sistema imunológico materno, destacando a importância de elucidar os mecanismos envolvidos durante o início da prenhez. Além disso, neste momento, o IFN- $\tau$  é liberado na circulação sanguínea e estimula a expressão de genes (ISGs). O estudo da expressão gênica dos ISGs clássicos (*ISG15*, *OAS1*, *MX1* e *MX2*) em células imunes do sangue periférico, como células mono (PBMC) e polimorfonucleares (PMN) já vem sendo utilizado como ferramenta de diagnóstico precoce da prenhez, porém apresentou baixa acurácia. Neste contexto, nossos objetivos neste estudo foram: 1) caracterizar a magnitude da resposta dos ISGs clássicos (*ISG15*) e não clássicos (*RSAD2* e *IFI44*); bem como o perfil das citocinas pró- (*IL1 $\beta$* ) e anti-inflamatórias (*IL10*) em PBMC e PMN estimuladas com IFN- $\tau$  (**Experimento 1**) ou lavados uterinos (UF) de vacas gestantes (**Experimento 2**); e 2) avaliar a acurácia desses biomarcadores recentemente identificados (*RSAD2* e *IFI44*) como preditores da prenhez precoce em fêmeas bovinas (**Experimento 3**). Nos Experimentos 1 e 2, PBMC e PMN foram isoladas do sangue de vacas Nelore não gestantes (N=9) entre 10-12 dias pós-ovulação (D0 = dia da ovulação), e estimuladas com 100 ng/mL de roIFNT ou UF de vacas no dia 18 de gestação. A expressão gênica foi determinada por qPCR. Para o Experimento 3, fêmeas Nelore (núlparas, N=103; primíparas, N=53; pluríparas, N=91) foram submetidas à coleta de

sangue para isolamento das PMN, e foi realizada ultrassonografia Doppler para avaliar a funcionalidade do CL no D20 após inseminação artificial em tempo fixo (D0 = dia da IATF). A expressão gênica também foi determinada por qPCR. Além disso, foram realizadas curvas ROC para determinar a acurácia dos preditores da prenhez no D20 (ISGs e Doppler). Nos Experimentos 1 e 2, a expressão de todos os ISGs foi maior ( $P < 0,05$ ) em ambas as células tratadas com roIFNT e UF-Conceptus do que nos grupos Controle. O fold change indicou que o *ISG15* e *RSAD2* foram os genes mais estimulados ( $P < 0,05$ ) em PBMC e PMN. A expressão de *IL1 $\beta$*  foi menor ( $P < 0,05$ ) em PBMC e PMN tratadas com UF-Conceptus; no entanto, nenhuma diferença ( $P > 0,1$ ) foi observada entre os outros grupos de tratamento. Para o Experimento 3, todas as combinações genéticas foram testadas, e a melhor associação para aumento da acurácia (92,7%) e redução de resultados falsos negativos (0,9%, 2/233) foi obtida quando os animais foram considerados prenhes se um dos os quatro ISGs (*ISG15*, *OAS1*, *RSAD2* e *IFI44*) foram estimulados em fêmeas com CL ativo ( $> 25\%$  de perfusão sanguínea) no D20. Concluindo, o *ISG15* e *RSAD2* foram os ISGs mais estimulados em PBMC e PMN, indicando que uma associação entre ISGs clássicos e não clássicos pode ser utilizada como uma ferramenta precoce para melhorar a predição da prenhez no D20 em fêmeas bovinas com CL ativo determinado através da US-Doppler.

**Palavras-chave:** ISG, células imunes, interferon- $\tau$ , lavado uterino, diagnóstico de prenhez.

## ABSTRACT

FELTRIN, I.R. **Study of genes stimulated by interferon- $\tau$  in immune cells as candidates for pregnancy markers in bovine females.** [Estudo de genes estimulados por interferon- $\tau$  em células imunes como candidatos à marcadores de prenhez em fêmeas bovinas]. 2024. 99 f. Tese (Doutorado em Farmacologia e Biotecnologia) – Instituto de Biociências, Universidade Estadual Paulista, Botucatu, 2024.

In bovine females, embryonic losses occurring between the 14th and 19th day after estrus significantly affect the reproductive efficiency of herds. Therefore, the development of diagnostic methods capable of early detection of pregnancy or non-pregnancy is an important point for improving the reproductive performance of cattle. Interferon- $\tau$  (IFN- $\tau$ ) produced by the conceptus plays an important role during maternal recognition of pregnancy (MPR) in ruminants, which involves more than the inhibition of luteolytic pulses of prostaglandin F2 alpha (PGF<sub>2 $\alpha$</sub> ) to maintain the function of corpus luteum (CL). For a successful pregnancy, the semi-allogeneic conceptus must avoid rejection by the maternal immune system, highlighting the importance of elucidating the mechanisms involved during early pregnancy. Furthermore, at this moment, IFN- $\tau$  is released into the blood circulation and stimulates the expression of genes (ISGs). The study of gene expression of the classic ISGs (*ISG15*, *OAS1*, *MX1*, and *MX2*) in peripheral blood immune cells, such as mono (PBMC) and polymorphonuclear (PMN) cells has already been used as a form of early diagnosis of pregnancy, but showed low accuracy. In this context, our objective were: 1) characterize the response magnitude of classic (*ISG15*) and non-classic (*RSAD2* and *IFI44*) ISGs; as well as pro- (*IL1 $\beta$* ) and anti-inflammatory (*IL10*) cytokines in PBMC and PMN stimulated by IFN- $\tau$  (**Experiment 1**) or uterine flush (UF) from pregnant cows (**Experiment 2**); and 2) evaluate the accuracy of these recently identified biomarkers (*RSAD2* and *IFI44*) as early pregnancy predictors in bovine females (**Experiment 3**). In the *Experiment 1 and 2*, PBMC and PMN were isolated from the blood of non-pregnant Nelore cows (N=9) between 10-12 days post-ovulation (D0 = day of ovulation) and stimulated with 100 ng/mL of roIFNT or UF from day 18 of pregnancy cows. Gene expression was determined by qPCR. For the *Experiment 3*, Nelore females (nulliparous, N=103;

primiparous, N=53; pluriparous, N=91) were submitted to blood collection for PMN isolation, and Doppler ultrasonography was performed to assess the functionality of the corpus luteum (CL) on D20 after timed-artificial insemination (D0 = TAI day). Gene expression was also determined by qPCR. In addition, ROC curves were performed to determine the accuracy of pregnancy predictors on D20 (ISGs and Doppler). In *Experiments 1 and 2*, expression of all ISGs was greater ( $P < 0.05$ ) in both cells treated with roIFNT and UF-Conceptus than its controls. The fold change indicated that *ISG15* and *RSAD2* were the most stimulated genes ( $P < 0.05$ ) in PBMC and PMN. Expression of *IL1 $\beta$*  was lesser ( $P < 0.05$ ) in PBMC and PMN treated with UF-Conceptus; however, no difference ( $P > 0.1$ ) was observed between the other treatment groups. For the *Experiment 3*, all gene combinations were tested, and the best association for an increase in accuracy (92.7%) and reduction of false negative results (0.9%, 2/233) was obtained when pregnant animals were considered if one of the four ISGs (*ISG15*, *OAS1*, *RSAD2*, and *IFI44*) were stimulated in females with an active CL (> 25% blood perfusion) on D20. In conclusion, *ISG15* and *RSAD2* were the most stimulated ISGs in PBMC and PMN, indicating that an association between classic and non-classic ISGs can be used as an early tool to improve pregnancy prediction at D20 in bovine females with active CL determined by US-Doppler.

**Keywords:** ISG, immune cells, interferon- $\tau$ , uterine flush, pregnancy diagnosis.

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## 1           **1. GENERAL INTRODUCTION**

2           Brazil has the second-largest cattle herd in the world, totaling around 202 million  
3 animals, representing 12.18% of the global herd (ABIEC, 2023). Although the Brazilian  
4 beef industry is on the rise, the reproductive efficiency of herds is still low and deserves  
5 attention. For example, the calving interval in Brazilian beef cattle operations is around  
6 16.3 months, far from the ideal calving period of 12-13 months (BARUSELLI et al.,  
7 2016). Therefore, developing and improving technologies that help increase the produc-  
8 tivity of properties and optimize breeding systems and herd profitability are essential.

9           To achieve enhanced genetic and production gains, reproductive strategies must  
10 prioritize improving service rates and reducing the interval between inseminations,  
11 without compromising the viability of the already-established pregnancy (SÁ FILHO et  
12 al., 2014). Based on this concept, protocols for resynchronizing ovulation in females who  
13 did not become pregnant were developed. Thus, the success in reducing prolonged cal-  
14 ving intervals is associated with an early pregnancy diagnosis combined with an efficient  
15 resynchronization protocol. In this context, early detection of embryonic loss is essential  
16 - which occurs between days 8 and 17 of pregnancy in 40% of cases (THATCHER et al.,  
17 2001) - or early detection of conception failure.

18           Embryonic losses significantly affect reproductive efficiency and result from fai-  
19 lure in maternal pregnancy recognition (MPR). MPR occurs between days 15 and 19 post-  
20 ovulation in bovine females, through the release of the interferon- $\tau$  (IFN- $\tau$ ), which is pro-  
21 duced and secreted by the trophoblastic cells of the conceptus in the uterine environment,  
22 which blocks the mechanisms involved in pulsatile secretion of prostaglandin F2 alpha  
23 (PGF<sub>2 $\alpha$</sub> ) by the endometrium (MARQUES et al., 2007). IFN- $\tau$  increases on day 15, peaks  
24 on day 20, and decreases to basal concentrations near day 25 (BAZER et al., 2009). In  
25 addition, IFN- $\tau$  acts on endometrial cells downregulating the estradiol (ER) and oxytocin

26 (OXTR) receptors (SPENCER et al., 2007). As a consequence, the pulsatile release of  
27  $\text{PGF}_{2\alpha}$ , induced by the binding of oxytocin (OT) to its endometrial receptor, does not  
28 occur, and the corpus luteum (CL) remains active producing high concentrations of pro-  
29 gesterone (P4) to maintain pregnancy.

30         Among the pregnancy diagnosis methods applied to cattle, the most used techni-  
31 que is B-mode ultrasonography between days 28 and 32 after timed artificial insemination  
32 (TAI) (MARQUES et al, 2012; STEVENSON et al., 2003). However, this method makes  
33 it impossible to identify non-pregnant cows immediately after MRP and before returning  
34 to estrus (FORDE et al., 2011). Actually, other techniques have become available for the  
35 diagnosis of pregnancy, one of which is the detection of structural luteolysis in non-preg-  
36 nant females using Doppler ultrasonography (Doppler-US). The detection of structural  
37 luteolysis has been used in beef cows between 20 and 22 days after TAI (PUGLIESI et  
38 al., 2014) with accuracy and sensitivity exceeding 90%. However, this technique has a  
39 limiting factor: the high number of false positives.

40         In the last years, advances have been achieved, generating the possibility of per-  
41 forming an indirect pregnancy diagnosis through the expression of genes stimulated by  
42  $\text{IFN-}\tau$  (ISGs) on days 18 and 20 of pregnancy in peripheral blood immune cells. The most  
43 traditional ISGs used to detect pregnancy in immune cells are Ubiquitin-like Modifier 15  
44 (*ISG15*), 2'-5'-Oligoadenylate Synthetase 1 (*OAS1*), MX dynamin as GTPase 1 (*MX1*),  
45 and MX dynamin as GTPase 2 (*MX2*) (KIZAKI et al. al., 2013; PUGLIESI et al., 2014).  
46 The increase of ISGs abundance in peripheral blood mononuclear cells (PBMC) has been  
47 reported in ewes (ANTONIAZZI et al., 2013), dairy cows (SHIRASUNA et al., 2012,  
48 FERRAZ et al., 2021), and beef cattle (PUGLIESI et al., 2014; DALMASO DE MELO  
49 et al., 2020). The expression of ISGs follows the  $\text{IFN-}\tau$  release pattern, which may indi-

50 cate pregnancy earlier than other conventional diagnostic methods. Furthermore, expres-  
51 sion of classic ISGs has also been reported in peripheral blood polymorphonuclear cells  
52 (PMN) in cattle (KIZAKI et al., 2013; TOJI et al., 2017; MELO et al., 2020), and there  
53 is evidence that PMNs are more sensitive to IFN- $\tau$  stimulation than PBMCs (KIZAKI et  
54 al., 2013).

55 The expression of classic ISGs in PBMC and PMN has been used in a few studies  
56 for prospective diagnosis of pregnancy on the 20th day post-TAI in beef cows; however,  
57 the maximum accuracy achieved was 87% (PUGLIESI et al., 2014; DALMASO de  
58 MELO et al., 2020). Therefore, in a previous study conducted by Rocha et al. (2020) using  
59 RNA sequencing, novel early-pregnancy-induced genes were identified in PBMC (*IFI6*,  
60 *RSAD2*, *IFI44*, *IFITM2*, *CLEC3B*, *OAS2*, *TNFSF13B*, *DMKN* and *LGALS3BP*) and  
61 PMN (*IFI44*, *RSAD2*, *OAS2*, *LGALS3BP*, *IFI6* and *CIR*) on the 18th day after TAI.  
62 These genes have the potential to be used as pregnancy biomarkers, offering an alterna-  
63 tive for greater accuracy in the early diagnosis of pregnancy compared to the classic ISGs  
64 already in used. Thus, a better understanding of the transcriptional stimulus of each bi-  
65 omarker in immune system cells is a necessary aspect to be explored to deepen the selec-  
66 tion criteria. Moreover, in the aforementioned study (Rocha et al., 2020), a limited sample  
67 size was used (N=6 animals/group), and as pregnancy assessment is a binary test, a larger  
68 number of animals must be explored to correctly select a pregnancy biomarker.

69 The herein study aims to determine novel pregnancy markers in peripheral blood  
70 immune cells that can serve as a basis for developing and improvement of molecular  
71 methods for pregnancy diagnosis in cattle. Our central hypothesis is that biomarkers in  
72 peripheral blood, stimulated by the presence of the conceptus, may be detectable at the  
73 early stages of pregnancy in circulating immune cells. This condition would enable the  
74 development of a method for early pregnancy diagnosis in bovine females 20 days after

75 mating. To test our hypothesis, the specific objectives of the current study were: 1) char-  
76 acterize the response magnitude of classic (*ISG15*) and non-classic (*RSAD2* and *IFI44*)  
77 ISGs; as well as pro- (*IL1 $\beta$* ) and anti-inflammatory (*IL10*) cytokines in PBMC and PMN  
78 stimulated by IFN- $\tau$  (*Experiment 1*) or uterine flush (UF) from pregnant cows (*Experi-*  
79 *ment 2*); and 2) evaluate the accuracy of these recently identified biomarkers (*RSAD2* and  
80 *IFI44*) as early pregnancy predictors in bovine females (*Experiment 3*).

81 This thesis is divided into two chapters. Chapter 1 reviews relevant topics related  
82 to the experiments conducted in the subsequent chapter. Chapter 2 describes the magni-  
83 tude of the transcriptional response of each ISG and cytokine due to challenge by direct  
84 stimulation of IFN- $\tau$  or UF in PBMC and PMN, using an *in vitro* study model consisting  
85 of two experiments. Based on the results obtained in these studies, we conducted the third  
86 experiment. In the third experiment, we tested the accuracy of ISGs expression in PMN  
87 as early pregnancy predictors in a large number of animals. Chapter 2 was written in  
88 accordance with the standards of the journal *Biology of Reproduction*, to which the man-  
89 uscript will be submitted. The final considerations at the end of this thesis summarize the  
90 most important findings of each experiment and discuss future perspectives for further  
91 studies.

92 We believe that the present study introduces an innovative aspect as it corresponds  
93 to the development of new techniques to diagnose pregnancy earlier than current meth-  
94 ods, in addition to generating the possibility of identifying new molecular markers in  
95 peripheral blood immune cells, in addition to classic ISGs. Such advances can directly  
96 affect the efficiency of reproductive programs in cattle, especially those that use TAI.

97  
98  
99

## 2. CHAPTER 1: LITERATURE REVIEW

### 2.1 PRODUCTION OF IFN- $\tau$ BY EMBRYO AND BOVINE CONCEPTUS

In ruminants, the establishment of pregnancy depends on the signals sent by the conceptus, which begins during the first weeks of pregnancy (HANSEN et al., 2017), followed by implantation and placentation. The bovine embryo begins its development in the ampulla of the oviduct. Around the 5th day after estrus, the embryo enters the uterine cavity, where it reaches, sequentially, the morula (4-7 days of development) and blastocyst stage (7-12 days of development) (SPENCER et al., 2007). The blastocyst, around the 9th-11th day, hatches in the zona pellucida, assuming an ovoid format. From this moment, the conceptus (embryo and placental membranes) undergoes intense and progressive elongation, achieved by the rapid multiplication of trophoctoderm cells (WANG et al., 2009).

The bovine conceptus secretes molecules that prevent the luteolysis to allow for the establishment of pregnancy, being IFN- $\tau$  secretion by the mononuclear cells of the trophoctoderm the main one (ROBERTS et al., 2008). The peak of IFN- $\tau$  production is reached on the 15th day of development in sheep and the 20th day in bovine females (GUILLOMOT et al., 1990). In sheep, small quantities of IFN- $\tau$  are produced in blastocysts at 8 to 10 days of development (ASHWORTH; BAZER, 1989), reaching maximum production between 14 and 16 days, and declining to undetectable concentrations at 25 days of development (BAZER et al., 1992; ROBERTS et al., 1999). In bovine, Sponchiado et al. (2017) suggest that the release of IFN- $\tau$  begins at the blastocyst stage, on the 7th day of development. The expression of IFN- $\tau$  ends with the beginning of embryo implantation at 25 days of pregnancy, as the contact of the trophoblast with the endometrium interrupts its production (DEMMERS et al., 2001).

124 IFN- $\tau$  is a protein formed by 172 amino acids and is only present in ruminants.  
125 The expression of the IFN- $\tau$  gene begins in the embryo, regardless of whether it is in the  
126 uterine cavity or not, since the expression is evident in embryos cultured *in vivo* and *in*  
127 *vitro* systems. However, the production of IFN- $\tau$  is influenced by the uterine environment,  
128 considering that embryos cultured *in vitro* increase the production of IFN- $\tau$  when exposed  
129 to the uterine environment (KERBLER et al., 1997).

130

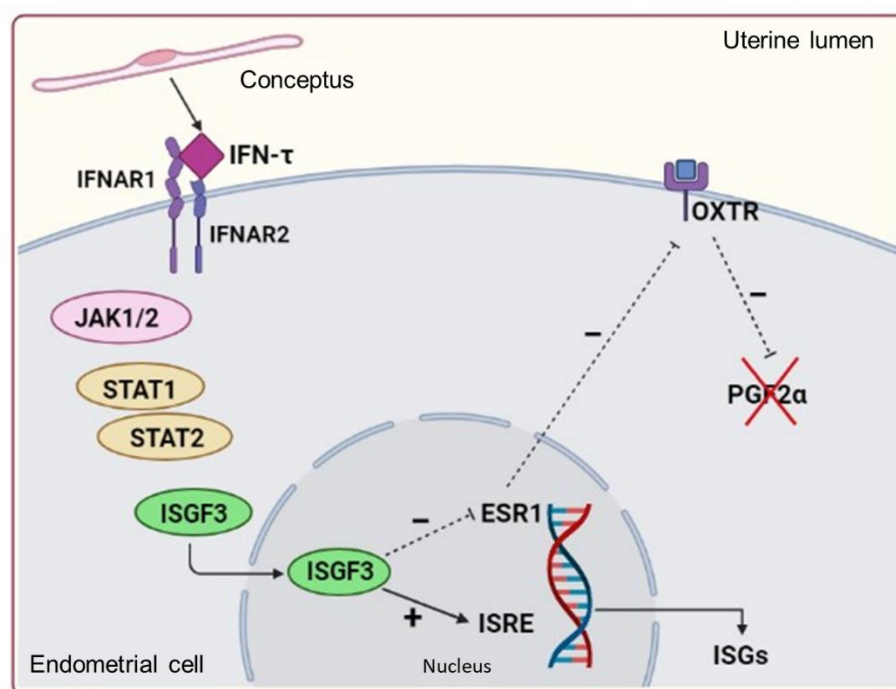
## 131 **2.2 AUTOCRINE AND PARACRINE EFFECTS OF IFN- $\tau$ DURING MA-** 132 **TERNAL RECOGNITION OF PREGNANCY**

133 IFN- $\tau$  can act on the MRP in ruminants as an important autocrine factor in the  
134 regulation of proliferative cells of the ovine trophoctoderm, however, this signaling is not  
135 well understood due to the difficulty in isolating the autocrine and paracrine effects. The  
136 autocrine action of IFN- $\tau$  was suggested by Wang et al. (2013), when they observed that  
137 sheep trophoctoderm cells cultured *in vitro* with 10, 100, or 1000 ng of recombinant bo-  
138 vine IFN- $\tau$  (rbIFNT) showed an increase in cell proliferation and expression of ISGs  
139 (*ISG15* and *OAS1*), with such effects being dose-dependent on the amount of rbIFNT.

140 The paracrine action of IFN- $\tau$  begins when it is released into the uterine lumen  
141 and connects to specific receptors (IFNAR), located on the membrane of endometrial  
142 cells. These receptors are formed by two subunits: IFNAR1 and IFNAR2. Once activated,  
143 IFNAR signaling can stimulate canonical (more traditional) or non-canonical pathways.  
144 The canonical signaling pathway involves Janus Kinase (JAK)-STAT-IRF which results  
145 in the prevention of luteolysis and expression of ISGs. In contrast, the non-canonical  
146 pathway is not well described and is uncommon (HANSEN et al., 2017); and it is acti-  
147 vated by mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase  
148 thymoma viral proto-oncogene 1 (PI3KAKT1) (STARK et al., 1998). The activation of

149 the JAK/STAT signaling cascade results in the formation of the transcription factor  
 150 ISGF3, which is transported to the cell nucleus, where it inhibits the transcription of en-  
 151 dometrial estradiol receptors (ESR) and, consequently, the endometrial oxytocin recep-  
 152 tors (OXTR). The absence of OXTR prevents oxytocin (OT) from connecting to its re-  
 153 ceptors, thus inhibiting the synthesis and occurrence of endometrial PGF<sub>2</sub> $\alpha$  pulses, and  
 154 preventing luteolysis (HANSEN et al., 2017).

155 The most common IFN- $\tau$  activated IRFs in the uterus are IRF1 and IRF2, which  
 156 have important roles in preventing luteolysis events (SPENCER et al., 1998). Therefore,  
 157 the IRF2 is important to inhibit OXTR expression independent of ESR expression in bo-  
 158 vine endometrium, while in sheep the mechanism is only related to ESR downregulation,  
 159 without effects on OXTR expression (HANSEN et al., 2017). In addition to preventing  
 160 luteolysis, IFN- $\tau$  stimulates the expression of classic and non-classic ISGs in the endo-  
 161 metrium (HANSEN et al., 2017), which is important for uterine receptivity (OKUMU et  
 162 al., 2011) and prevents immune rejection of the conceptus (CHOI et al., 2003). The para-  
 163 crine actions described in this section are represented in **Figure 1**.



164

165 **Figure 1.** Paracrine actions of IFN- $\tau$ . The IFN- $\tau$  produced by the conceptus is secreted  
166 into the uterine lumen and connects to type I interferon receptors (IFNAR1/IFNAR2),  
167 activating the JAK/STAT signaling pathway. Once the cascade is activated, the transcrip-  
168 tion factor ISGF3 is formed, which is translocated to the nucleus of the endometrial cell.  
169 ISGF3 inhibits the transcription of the endometrial estradiol receptor (ESR1) and, conse-  
170 quently, the endometrial oxytocin receptor (OXTR). The lack of oxytocin (OT) signaling  
171 at its receptor inhibits the synthesis and release of PGF<sub>2 $\alpha$</sub> . ISGF3 connects to the inter-  
172 feron-responsive region (ISRE), initiating the synthesis of ISGs. Source: Feltrin, I. R.  
173

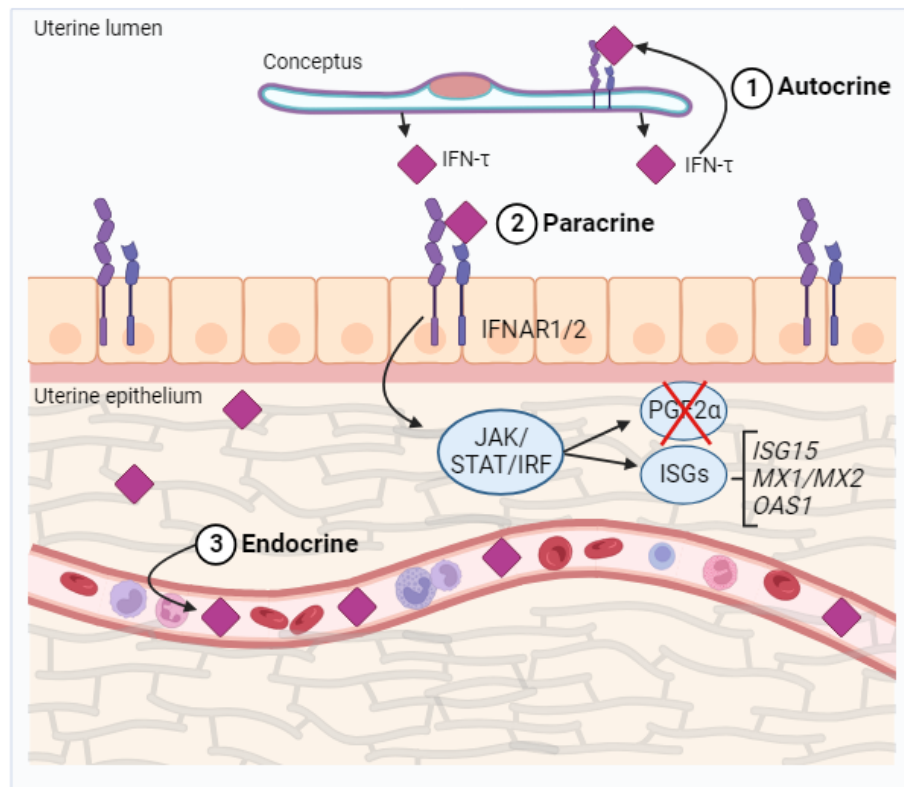
### 174 **2.3 ENDOCRINE EFFECTS OF IFN- $\tau$ ON THE CORPUS LUTEUM AND** 175 **IMMUNE CELLS DURING MATERNAL RECOGNITION OF PREGNANCY**

176 In addition to the actions already described, IFN- $\tau$  can exit the uterine lumen  
177 through the uterine vein and act on distant tissues in an endocrine manner. The endocrine  
178 effects of IFN- $\tau$  were confirmed in several studies, which showed the expression of ISGs  
179 in the liver (MEYERHOLZ et al., 2015), luteal cells (BRIDI et al., 2018), and immune  
180 cells in the bloodstream (PUGLIESI et al., 2014; DALMASO DE MELO et al., 2020).  
181 Furthermore, IFN- $\tau$  was also identified in extracellular vesicles secreted by the conceptus  
182 and released into the systemic circulation (NAKAMURA et al., 2016). Although IFN- $\tau$   
183 activity has been confirmed in several tissues, no antiviral activity has been detected in  
184 the main (jugular) vessels of the circulatory system. However, Bott et al. (2010) reported  
185 the presence of antiviral activity in uterine vein plasma in ewes on day 15 of pregnancy.

186 In this context, two hypotheses were formulated by Hansen et al. (2017) to eluci-  
187 date through which mechanisms IFN- $\tau$  exert its endocrine effects. The first hypothesis  
188 suggests that IFN- $\tau$  is a glycoprotein with potent action and rapid clearance in systemic  
189 circulation; thus, low concentrations of IFN- $\tau$  would be sufficient to exert its actions. This  
190 hypothesis is reinforced by the observations of Schalue-Francis et al. (1991), who found

191 only 58 antiviral units per mL of IFN- $\tau$  in the uterine vein. The second hypothesis sug-  
192 gests that IFN- $\tau$  could stimulate the expression of ISGs through extracellular vesicles,  
193 which would explain its relatively low concentration found in the peripheral circulation.  
194 IFN- $\tau$  has a high affinity for its receptors and a short plasma half-life of 7 to 9 hours  
195 (ZHAO et al., 2019). Thus, low circulating concentrations of IFN- $\tau$  would be sufficient  
196 to stimulate IFNAR (LI and ROBERTS, 1994). Recently, a new hypothesis was devel-  
197 oped in an attempt to elucidate the endocrine mechanisms by which IFN- $\tau$  reaches extra-  
198 uterine tissues. In this mentioned study, it was evidenced that embryo-derived IFN- $\tau$  on  
199 day 7 of development generates anti-inflammatory responses with upregulation of ISG  
200 expression in PMNs and that they subsequently amplify and transfer IFN- $\tau$  signals to a  
201 new PMN population through the communication mechanism cell-cell (FIORENZA et  
202 al., 2021).

203           The autocrine, paracrine, and endocrine effects of IFNT described in this review  
204 are represented in **Figure 2**.



205

206 **Figure 2.** Autocrine, paracrine, and endocrine actions of IFN- $\tau$ . 1) IFN- $\tau$  connects to type  
 207 I interferon receptors (IFNAR1/2) present in the trophoctoderm, exerting effects on the  
 208 conceptus itself; 2) IFN- $\tau$  produced by the conceptus, inhibits the transcription of endo-  
 209 metrial estradiol (ESR) and oxytocin (OXTR) receptors, inhibiting the synthesis of  
 210 PGF $_{2\alpha}$  in the endometrium and also induces the transcription of genes stimulated by IFN-  
 211  $\tau$  ( ISGs); 3) IFN- $\tau$  reaches the systemic circulation, from its exit through the uterine vein,  
 212 stimulating the expression of ISGs in extrauterine tissues. Source: Feltrin, I. R. (adapted  
 213 from Rocha et al., 2021).

214

215 The prevention of luteolysis by IFN- $\tau$  not only occurs through the inhibition of  
 216 pulsatile secretion of PGF $_{2\alpha}$  but also a luteoprotective and luteotrophic effect of IFN- $\tau$  in  
 217 the CL (ANTONIAZZI et al., 2013). For example, endocrine administration of roIFNT  
 218 for 24 hours, regardless of the route of administration (uterine or jugular veins) or dose  
 219 (200 or 20  $\mu$ g/day), effectively protects the CL against the decrease in P4 concentrations  
 220 induced by PGF $_{2\alpha}$  administration. i. m. in ewes. Furthermore, in response to IFN- $\tau$ , there

221 was downregulation of the main membrane transporter of prostaglandins, *SLCO2A*, and  
222 the main  $\text{PGF}_{2\alpha}$  receptor (*PTGFR*) in the CL tissue (BANU et al., 2008; ANTONIAZZI  
223 et al., 2013). In addition,  $\text{IFN-}\tau$  also generates upregulation of prostaglandin E synthase  
224 (*PTGES*) and downregulation of COX-2 in the CL, which modulates the production of  
225 luteoprotective  $\text{PGE}_2$  in the endometrium (AROSH et al., 2004). Therefore, we have three  
226 combined mechanisms that favor MRP, such as the inhibition of  $\text{PGF}_{2\alpha}$  synthesis, the  
227 greater resistance of the CL to the luteolytic actions of  $\text{PGF}_{2\alpha}$ , and the set of luteotrophic  
228 actions triggered by  $\text{IFN-}\tau$  and  $\text{PGE}_2$ .

229         The identification of increased expression of ISGs in luteal tissue has been ex-  
230 plained by the endocrine actions of  $\text{IFN-}\tau$  on the CL (OLIVEIRA et al., 2008). The ex-  
231 pression of *ISG15* in large luteal cells of ewes at 15 days of pregnancy was greater than  
232 in non-pregnant ewes. Still, in the same study,  $\text{IFN-}\tau$  concentrations were greater in the  
233 blood of the uterine artery, uterine vein, and jugular vein in pregnant females than in  
234 cyclic females (OLIVEIRA et al., 2008). Furthermore, in ewes that received the infusion  
235 of 200  $\mu\text{g}$  roIFNT, the expression of luteal *ISG15* was greater compared to the control  
236 group (BOTT et al., 2010). Likewise, bovine luteal cells treated *in vitro* with increasing  
237 doses of interferon 2 alpha ( $\text{IFN}\alpha$ , 0.1 - 100 ng/mL) showed increased *ISG15* expression  
238 in a dose-dependent manner, compared to the control group (BRIDI et al., 2018).

239         The expression of classic ISGs in immune cells has been extensively studied in  
240 ruminants over the years. Regardless of the cell type, PBMC or PMN, the expression  
241 profile is similar to the secretion of  $\text{IFN-}\tau$  by trophoblastic cells, where the expression of  
242 ISGs peaks on day 20 of pregnancy, and decreases to basal levels around day 25 (SHI-  
243 RASUNA et al., 2012; PUGLIESI et al., 2014). The amount of  $\text{IFN-}\tau$  released by the  
244 uterine horn correlates with the levels of *ISG15* expression in immune cells (MATSU-  
245 YAMA et al., 2012). These results corroborate the positive correlation found between the

246 expression of ISGs in immune system cells and the concentration of exogenous IFN- $\tau$   
247 administered (MATSUYAMA et al., 2012). One study evaluated ISG expression in  
248 whole blood from the uterine vein, uterine artery, and jugular vein; however, no differ-  
249 ences were found in the expression of *ISG15* and *OAS1* regardless of the type of blood  
250 vessel (OLIVEIRA et al., 2008). Thus, immune cells can be stimulated when they enter  
251 the uterine vein, corroborating the hypothesis that cells transfer signals between them-  
252 selves.

253         It is known that immune cells play an important role during MRP, preventing the  
254 rejection of the semi-allogeneic conceptus, however, the functions of each ISG in PBMC  
255 and PMN are still poorly understood. The infusion of PBMC into the uterine lumen of  
256 cyclic heifers, on the 4th day of the estrous cycle, improved the quality of embryos trans-  
257 ferred on day 7 and collected on day 15 (IDETA et al., 2010). As observed by Yang et al.  
258 (2016), immune cells from the bloodstream, which were isolated on the 18th day of preg-  
259 nancy, showed downregulation of genes involved in the luteolysis process, such as aldo-  
260 keto reductase family 1 member B (*AKR1B1*). These findings illustrate the role of im-  
261 mune cells in maintaining CL function.

262         Although IFNARs are present in the endometrial epithelium, stroma, and glands,  
263 the *ISG15* and *OAS1* genes are only expressed in stromal cells and endometrial glands,  
264 while *MX1* and *MX2* are only expressed in the luminal epithelium regardless of gesta-  
265 tional status. In this context, a series of studies demonstrate that regardless of the cell  
266 type, PBMC or PMN, the expression of mRNA for the classic ISGs (*ISG15* and *OAS1*)  
267 was higher in pregnant cows compared to non-pregnant cows, on days 18 and 20 after  
268 artificial insemination (AI) (GREEN et al., 2010; PUGLIESI et al., 2014; DALMASO de  
269 MELO et al., 2020; ROCHA et al., 2020).

270

271           **2.4 MODULATION OF THE IMMUNE SYSTEM AT THE ONSET OF**  
272 **PREGNANCY**

273           The embryo is a semi-allogeneic structure, as it contains half of the DNA origi-  
274 nating from the paternal individual (FAIR, 2015). Thus, for successful MRP, both the  
275 conceptus and the maternal immune system act in synergy to allow pregnancy to be es-  
276 tablished. The cow's immune system is formed by two main components: the adaptive  
277 immune system (acquired immunity) and the innate immune system (natural immunity).  
278 The innate immune system has been primarily responsible for the immunological changes  
279 observed during MRP, as it uses receptors and cells to detect pathogens, such as Toll-like  
280 receptors (TLR), complement system, and natural killer (NK) cells (HOEBE et al., 2004).

281           During early pregnancy, two immunological events prevent rejection of the semi-  
282 allogeneic conceptus. The first event begins during mating, when the semen is exposed  
283 to the female reproductive system, and induces the expression of immune modulators,  
284 including interleukins (EZZ et al., 2019). Sperm-uterus communication generates a pro-  
285 inflammatory immune response, which is necessary to remove excess dead sperm and  
286 possible invading pathogens, which enter the reproductive tract during mating or insem-  
287 ination. Contrary, sperm-oviduct communication generates an anti-inflammatory im-  
288 mune response, necessary for the survival of sperm until fertilization occurs (TALUK-  
289 DER et al., 2020). The second event occurs when the conceptus trophoblastic cells begin  
290 contact with the maternal endometrium (FAIR et al., 2015). The immunological interac-  
291 tion between the bovine conceptus and the endometrium, during the elongation period,  
292 after blastocyst hatching (12th and 13th days after estrus), and in the MRP period (16th  
293 to 19th days after estrus) has already been extensively investigated (OLIVEIRA et al.,  
294 2013; KAMAT et al., 2016; VASUDEVAN et al., 2017). However, few studies have been

295 conducted focusing on uterine immunological responses due to the presence of the em-  
296 bryo during the pre-hatching period of blastocysts in cattle. Recently, Talukder et al.  
297 (2017), using *in vitro* models, demonstrated that embryos at the morula stage (5th day of  
298 development) and blastocyst (9th day of development) induced the expression of ISGs  
299 (*ISG15*, *OAS1*, *MX2*), transcription factors (*STAT*), and receptors (*IFNAR1* and *IFNAR2*)  
300 on the epithelial and immune cells of the uterus.

301 The first studies that attempted to elucidate the mechanisms by which the allogeneic  
302 conceptus survives the maternal immune system in vertebrates were developed by  
303 Medawar (1953), who proposed three possible mechanisms: 1°) the conceptus is inert and  
304 does not express histocompatibility antigens; 2°) there would be immunosuppression of  
305 the maternal immune system; and 3°) the placenta would act as a protective barrier be-  
306 tween the conceptus and maternal immune cells. Regarding the first hypothesis, recent  
307 studies in cattle have demonstrated that major histocompatibility complex class I (*MHC-*  
308 *I*) genes are expressed during the development of the blastocyst (DOYLE et al., 2009)  
309 and trophoblastic cells, during the second half of pregnancy (DAVIES; FISHER;  
310 SCHLAFER, 2000). MHC-I is responsible for the potential antigenicity and the probable  
311 cause of the early response of endometrial and dendritic phagocytic cells (DOYLE et al.,  
312 2009). Thus, these results do not corroborate the hypothesis that the conceptus would be  
313 an immunologically inert structure.

314 Regarding the second hypothesis, maternal immunomodulation is associated with  
315 gestational success in cattle; however, the number of studies on this subject is still scarce.  
316 Evidence suggests that IFN- $\tau$  can modulate the immune function of the uterus, stimulat-  
317 ing immunosuppressive molecules and genes that regulate uterine receptivity and embry-  
318 onic development. In a study conducted by Mansouri-Attia et al. (2012), it was found that  
319 the expression of ISGs, such as *TNF $\alpha$* , *ISG15*, *IL12B*, *PTX3*, chemoattractant protein of

320 monocytes 1 (*MCP1*) and 2 (*MCP2*) was increased in the endometrial tissue of cattle.  
321 MCP1 and MCP2 proteins are potent chemotactic factors for monocytes, which are one  
322 of the most important cell types during the maternal immune response to the embryo in  
323 cattle (VELÁZQUEZ et al., 2019). The maternal innate immune response is also regu-  
324 lated by the embryo, evidenced by a downregulation of interleukin 1 $\beta$  (*IL1 $\beta$* ) and the  
325 nuclear factor- $\kappa$  $\beta$  (*NF- $\kappa$  $\beta$* ) system (MUÑOZ et al., 2012).

326 In addition, during pregnancy, immunological patterns are modified by signaling  
327 from the conceptus, and these patterns can alternate between a pro- or anti-inflammatory  
328 state. Therefore, another important immunological component, strongly modulated in the  
329 uterus, are lymphocytes, through T helper cells (Th) (MAEDA et al., 2013). These cells  
330 modulate the balance between Th1 and Th2 responses, which are balanced according to  
331 the cytokines secreted. Th1 cells generate cytokines such as IFN- $\gamma$ , IL1 $\beta$ , and tumor ne-  
332 crosis factor-alpha (TNF $\alpha$ ). Th2 cells produce IL4, IL5, IL6, IL10, and IL13 and nega-  
333 tively regulate Th1 responses (MOSMANN et al., 1986). In this context, trophoblastic  
334 cells cultured *in vitro* and treated with Th1 cells had their growth reduced (BERKOWITZ  
335 et al., 1988). In contrast, cytokines such as IL12, released by the Th2 immune response,  
336 induced conceptus tolerance in mice (LI et al., 1998). Thus, Th2 cytokines contribute to  
337 pregnancy success, while Th1 cytokines in the endometrium are unfavorable to MRP. In  
338 contrast, other studies have demonstrated a Th1-type environment in healthy pregnancies  
339 (GERMAIN et al., 2007; GUPTA et al., 2005). Therefore, more studies are still needed  
340 to elucidate the mechanisms by which the conceptus modulates immunological patterns  
341 to avoid its rejection during early pregnancy in cattle. In summary, there is no immuno-  
342 suppression of the maternal immune response and the conceptus is not immunologically  
343 inert, due to fetal expression of histocompatibility antigens. Although some immune func-  
344 tions are suppressed in the uterus during pregnancy, there is little evidence that pregnancy

345 leads to immunosuppression in the mother (OLIVEIRA et al., 2012; OTT et al., 2014).  
346 However, when pregnancy occurs, there is a different stimulation of the immune system,  
347 which, as opposed to host-pathogen interactions, involves immunological changes related  
348 to both the activation and suppression of immune functions (VELÁZQUEZ et al., 2019).

349         The last hypothesis proposed by Medawar (1953) is based on the possibility that  
350 the placenta is a barrier between the conceptus and maternal immune cells. The placenta  
351 is an organ formed by maternal and fetal tissues, responsible for exchanges between both  
352 components. Ruminants have an epithelial-chorial type placenta; thus, the binucleated  
353 cells of the trophoctoderm begin to fuse with the epithelial tissue and, probably, beyond  
354 it, from the 19th day of pregnancy. This fusion generates maternal-fetal hybrids, which  
355 are abundant in placentomes. Thus, the uterus, although it has populations of T cells suf-  
356 ficient to induce rejection of the conceptus (HANSEN et al., 1986), presents a decrease  
357 in the number of lymphocytes when placentation begins, especially in the placentomes.  
358 This mechanism is mediated by cells from the placenta itself, to protect the conceptus  
359 from attack by the immune system (LEE et al., 1997). Therefore, according to the anat-  
360 omy-physiological characteristics of the ruminant placenta, these results do not corrobo-  
361 rate the hypothesis that the placenta is a barrier between the mother and the fetus.

362

## 363           **2.5 USE OF ISGS IN EARLY DIAGNOSIS OF PREGNANCY IN BOVINE** 364 **FEMALES**

365         Pregnancy diagnosis (PD) is an essential part of activities that involve the repro-  
366 ductive management of beef and dairy cattle. After the use of TAI, mated females who  
367 did not become pregnant must be submitted to resynchronization in a shorter interval  
368 (PUGLIESI et al., 2019). Therefore, the development of early pregnancy detection meth-

369 ods becomes essential to reduce the interval between two consecutive TAI, conse-  
370 quently, reducing the prolonged calving intervals. Among the PD methods used in cattle,  
371 the gold standard method is B-mode transrectal ultrasound, performed with high accuracy  
372 between 28 and 32 days after TAI, by visualizing the embryo or fetus with a heartbeat  
373 (MARQUES et al, 2012). This method, however, does not allow for earlier management  
374 decisions to be made before or close to the time of normal return to estrus. More recently,  
375 the use of Doppler-US began to be used as an important tool for early PD, in which preg-  
376 nancy is confirmed indirectly through the assessment of CL blood perfusion, between  
377 days 20 and 22 of pregnancy, with accuracy greater than 90% (PUGLIESI et al., 2014,  
378 2018; MOTTA et al., 2020). One of the main advantages of this technique is its 100%  
379 sensitivity, which results in no false negative diagnoses. However, it results in 15 to 20%  
380 of false positive diagnoses, which may be due to delayed ovulation in response to the  
381 synchronization protocol, prolonged estrous cycles lasting longer than 22 days, or the  
382 occurrence of early embryonic mortality before PD (PUGLIESI et al., 2018).

383         In recent years, several research groups have sought to develop biomolecular  
384 methods that are capable of early detection of pregnancy or MRP failures. Thus, the quan-  
385 tification of ISGs in peripheral blood leukocytes was recently reported as an alternative  
386 method to indirectly detect the presence of the conceptus earlier than other traditional  
387 methodologies applied in cattle (GIFFORD et al., 2007; GREEN et al., 2010; YOSHINO  
388 et al., 2018). Most studies identified day 18 or 20 of pregnancy as the best time to compare  
389 the expression of classic ISGs (*ISG15*, *MX1*, *MX2*, and *OAS1*) between pregnant and non-  
390 pregnant cows (GREEN et al., 2010; SHIRASUNA et al., 2012; PUGLIESI et al., 2014).  
391 This is due to the small overlap of data at this time resulting from the greater secretion of  
392 IFN- $\tau$  around the 18-20th day of pregnancy, which increases proportionally to the growth  
393 of the trophoctoderm (THATCHER et al., 1995; ROBERTS et al. 2008). In addition, the

394 expression profile of ISGs is maintained between studies and regardless of status and  
395 breed.

396 The expression of ISGs in immune cells was tested as a prospective method for  
397 pregnancy diagnosis between days 20-22 in beef heifers and cows (PUGLIESI et al.,  
398 2014; DALMASO DE MELO et al., 2020), and dairy cows (FERRAZ et al., 2021;  
399 YOSHINO et al., 2018). The use of *MX2* and *OAS1* expression in PBMC on day 20 post-  
400 TAI in beef cows proved an accuracy of 62 to 80% to detect the presence of the conceptus,  
401 but sensitivity ranged from 66 to 78% (PUGLIESI et al., 2014). Similarly, the use of  
402 *ISG15* and *OAS1* expression in PMN on day 20 showed an accuracy of 72 to 81%, being  
403 greater in beef heifers than cows. Likewise, in dairy cows, Yoshino et al. (2018) reported  
404 an accuracy of 80% using the expression of *ISG15* and *MX2* in granulocytes between 20-  
405 22 days of pregnancy.

406 Considering the current results that indicate that Doppler-US is a highly effective  
407 tool for detecting non-pregnant females (100% sensitivity), and its main limitation is the  
408 number of false positive results; the combined use of this technique associated with the  
409 expression of classic ISGs was used in an attempt to increase the accuracy in pregnancy  
410 predicting. Therefore, Pugliesi et al. (2014) and Dalmaso de Melo et al. (2020) reported  
411 superior accuracy (84 to 90%) when combining the use of two classic ISGs (*OAS1/MX2*  
412 and *ISG15/OAS1*) in females with functional CL (blood perfusion > 25%) identified  
413 through Doppler-US on day 20 of pregnancy. Still, even combining the two methods,  
414 false positive and false negative results were not mitigated, leading to low accuracy;  
415 which in practical situations would cause financial losses to the producer, mainly due to  
416 false negative results. Therefore, a potential strategy to improve this accuracy and better  
417 understand immunological physiology during early pregnancy is to discover new tran-

418 scripts stimulated by the presence of a viable conceptus and use them as markers of preg-  
419 nancy in peripheral blood immune cells. In this context, Rocha et al. (2020) identified,  
420 through RNA sequencing, nine potential pregnancy biomarkers on PBMC (*IFI6*, *RSAD2*,  
421 *IFI44*, *OAS2*, *LGALS3BP*, *IFITM2*, *TNFSF13B*, *CLEC3B*, and *DMKN*) and five (*IFI6*,  
422 *RSAD2*, *IFI44*, *OAS2*, and *LGALS3BP*) on PMN on day 20 post-AI, which can serve as a  
423 basis for the development of a more accurate pregnancy prediction method.

424         The inaccuracy for predicting gestational status through ISGs is a result, in part,  
425 of the proportion of false positives that can occur because of early embryonic mortality,  
426 or the induction of ISGs by other types of stimuli. IFNAR is a non-selective receptor and  
427 can be stimulated by any type 1 IFN, which means that other IFNs can bind to it and  
428 stimulate the expression of ISGs, for example, in viral infections. Another important pa-  
429 rameter to be considered is the false negative rates, which must be zero for PD. In a study  
430 that used the expression of classic ISGs for early PD in cattle on the 20th day post-TAI,  
431 the false negative rate reached 30% (DALMASO de MELO et al., 2020). False-negative  
432 results may be related to animals in which IFN- $\tau$  does not signal sufficiently to stimulate  
433 the expression of ISGs. In this context, it is known that the size of the conceptus is directly  
434 correlated with the amount of IFN- $\tau$  released (SPENCER et al., 2016).

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803           **3. CHAPTER 2: BOVINE CONCEPTUS INDUCES ACCURATE BI-**  
804           **OMARKERS IN IMMUNE CELLS FOR EARLY PREGNANCY DIAG-**  
805           **NOSIS**

806

807           **3.1 INTRODUCTION**

808           In cattle, successful establishment of pregnancy depends on adequate communi-  
809 cation between the developing embryo/conceptus and the uterine environment during ma-  
810 ternal recognition of pregnancy (MRP) [1]. Interferon- $\tau$  (IFN- $\tau$ ), a trophoctoderm-derived  
811 cytokine, is regarded as a principal molecule responsible for the MRP in ruminants. Dur-  
812 ing MRP, IFN- $\tau$  inhibits the endometrial pulsatile release of prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ),  
813 preventing regression of the corpus luteum (CL), and maintaining the secretion of pro-  
814 gesterone (P4) necessary for the establishment of pregnancy [2, 3]. The successful occur-  
815 rence of this sequence of events determines gestational success; however, 50% of beef  
816 cattle fail to become pregnant by day 16 after artificial insemination (AI) [4]. For this  
817 reason, understanding the mechanisms involved in early pregnancy is essential to prevent  
818 pregnancy loss, and the maternal immune system plays an important role in this process.

819           Studies suggest that conceptus alloantigens alter maternal immune function both  
820 locally at the embryonic-maternal junction and systemically in the peripheral blood cir-  
821 culation, to prevent embryonic immune rejection. This is achieved through the modula-  
822 tion of maternal immune cells that direct the balance of cytokines towards the Th2 anti-  
823 inflammatory pathway [5]. This character of immunological tolerance displayed by im-  
824 mune cells can be triggered by several molecules such as hormones, cytokines, and en-  
825 zymes [6]. Thus, IFN- $\tau$  is the cytokine responsible for the functional connection between  
826 the maternal immune system and the developing embryo in ruminants. Recent studies

827 indicate that the bovine embryo on day 4 of development is already capable of synthesiz-  
828 ing IFN- $\tau$ , regulating the local immune environment in the oviduct [7]. Furthermore, bo-  
829 vine embryos at day 7 communicate with epithelial and immune cells, mediated in part  
830 by IFN- $\tau$  [8].

831 IFN- $\tau$  is also known to induce the expression of interferon-stimulated genes  
832 (ISGs) in the liver, endometrium, luteal cells, and peripheral blood mononuclear (PBMC)  
833 and polymorphonuclear (PMN) cells during early pregnancy in cows [9-12]. The genes  
834 commonly stimulated by IFN- $\tau$ , known as classic ISGs, are ubiquitin-like modifier 15  
835 (*ISG15*), 2'-5'-oligoadenylate synthetase 1 (*OAS1*), MX dynamin-like GTPase 1 (*MX1*)  
836 and 2 (*MX2*). The transcriptional profile of these genes is closely related to the secretion  
837 of IFN- $\tau$  by the conceptus [9-12]. In cattle, the peak expression of ISGs occurs between  
838 days 18 and 20 of pregnancy, assuming basal levels close to day 25. In addition, the  
839 mRNA expression for *ISG15* in bovine peripheral blood leukocytes is greater in pregnant  
840 than in non-pregnant cows on days 18 and 20 after AI. These findings suggest that IFN-  
841  $\tau$  can modulate the maternal immune system during early pregnancy. Such information  
842 generated the possibility of early detection of pregnancy or MRP failures through the  
843 expression of ISGs in immune cells in this period [12, 13].

844 After timed-AI (TAI), the females that did not become pregnant must be identified  
845 as soon as possible for a new service. However, the most common method of pregnancy  
846 diagnosis in cattle is the transrectal ultrasound in B-mode, which is performed with 100%  
847 accuracy between 28-32 days after TAI by visualizing the viable embryo. Therefore, con-  
848 sidering that non-pregnancy females return to estrus around 21 days after TAI [14], the  
849 development of methods for early detection of pregnancy ( $\leq 20$  days post-TAI) becomes  
850 essential to reduce the interval between two consecutive TAIs. Recently, the use of color-  
851 Doppler has been used for early pregnancy diagnosis, where pregnancy is confirmed by

852 maintaining CL blood perfusion between days 20 and 22 with an accuracy greater than  
853 90% [12, 15, 16]. The advantage of this technique is the sensitivity of 100%, which results  
854 in no false negative diagnoses; however, this method may result in up to 15% false-positi-  
855 tive diagnoses in beef, and 40% in dairy cattle [16, 17]. Thus, the development of methods  
856 capable of identifying pregnancy by detecting the conceptus or using conceptus-specific  
857 markers could reduce false positive rates and improve the accuracy of pregnancy diagno-  
858 sis methods during the first three weeks after TAI.

859         The expression of classic ISGs in immune cells has been evaluated as a prospec-  
860 tive diagnostic method for detecting pregnancy on day 20 in both heifers and cows. How-  
861 ever, the accuracy ranged from 62% to 80%, regardless of cell type (PBMC or PMN) [12,  
862 18]. In this context, Rocha et al. [19] identified, through RNA sequencing, new genes  
863 induced by early pregnancy in PBMC and PMN on day 18 post-TAI, which can be con-  
864 sidered potential biomarkers of pregnancy, as well as a potential strategy to increase the  
865 precision of the method compared to classic ISGs already used. Therefore, a comprehen-  
866 sive understanding of the transcriptional stimuli for each biomarker in immune system  
867 cells is a crucial aspect to explore, along with the inclusion of a larger number of animals  
868 to ensure the accurate selection of a pregnancy biomarker.

869         Therefore, our central hypothesis is that biomarkers in peripheral blood, stimu-  
870 lated by the presence of the conceptus, may be detectable at the early stages of pregnancy  
871 in circulating immune cells. This condition would enable the development of a method  
872 for early pregnancy diagnosis in bovine females 20 days after mating. The study aimed  
873 1) to characterize the magnitude response of classic (*ISG15*) and non-classic (*RSAD2* and  
874 *IFI44*) ISGs; as well as pro-*(IL1 $\beta$ )* and anti-inflammatory (*IL10*) cytokines in PBMC and

875 PMN stimulated by IFN- $\tau$  (*Experiment 1*) or uterine flush (UF) from pregnant cows (*Ex-*  
876 *periment 2*); and 2) evaluate the accuracy of these recently identified biomarkers (*RSAD2*  
877 and *IFI44*) as predictors of early pregnancy in bovine females (*Experiment 3*).

878

## 879 **3.2 MATERIAL AND METHODS**

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### 881 **3.2.1 Ethics statement**

882 The present study was conducted at the Animal Reproduction Department of the  
883 University of São Paulo, in Pirassununga, Brazil. All animals were in adequate health  
884 status and were handled under the guidelines of the Institutional Committee for Ethics in  
885 Research of the University of São Paulo, which also approved this study (CEUA-FMVZ  
886 no.: 8192280317).

887

### 888 **3.2.2 Experimental model**

889 Initially, to characterize the magnitude response of ISGs (*ISG15*, *RSAD2* and  
890 *IFI44*), as well as the profile of pro- (*IL1B*) and anti-inflammatory (*IL10*) cytokines,  
891 PBMC and PMN isolated from the peripheral blood of Nelore heifers were stimulated  
892 with 100 ng /mL recombinant ovine interferon- $\tau$  (roIFNT, *Experiment 1*) or UF from day  
893 18 of pregnant cows (*Experiment 2*) in an *in vitro* culture cell system. After the incuba-  
894 tion, the cells were analyzed for gene expression by quantitative PCR (qPCR), to deter-  
895 mine whether the responsiveness of non-classic ISGs (*RSAD2* and *IFI44*) to challenge  
896 with IFN- $\tau$ , resembled the response of already known classic ISGs (*ISG15*). Next, based  
897 on the responses obtained in the *in vitro* studies; the ISGs (*ISG15*, *OAS1*, *RSAD2* and  
898 *IFI44*) were tested for their accuracy in predicting gestational status in blood-isolated

899 PMN of different parity order categories (nulliparous, primiparous and pluriparous) on  
900 day 20 after TAI through gene expression by qPCR in an *in vivo* study (**Experiment 3**).

901

### 902 **3.2.3 Experimental design of Experiments 1 and 2**

903 Twelve Nelore beef heifers (*Bos taurus indicus*), non-pregnant, with a body con-  
904 dition score between 3 and 4 (scale 1–5) [20] and between 23 and 26 months, were used  
905 in this study. Animals were maintained on *Brachiaria brizantha* pastures with free access  
906 to water and mineral supplementation. On a random day of the estrous cycle, all animals  
907 received 2 mL i.m. of PGF<sub>2α</sub> (500 µg; of sodium cloprostenol; Sincrocio; Ouro Fino  
908 Saúde Animal) for estrous synchronization. In the following five days, the females were  
909 evaluated daily through ultrasound examinations to detect ovulation by the disappearance  
910 of the pre-ovulatory follicle. Between 10 and 12 days post-ovulation, CL blood perfusion  
911 was evaluated by color-Doppler ultrasound. A duplex B-mode (gray-scale) and pulse  
912 wave color-Doppler ultrasound instrument (MyLab Delta Vet Gold; Esaote Healthcare;  
913 Italy) equipped with a multifrequency linear transducer (3.5–7.5 MHz) in B-mode (RES-  
914 A, gain 50%, P 74 mm, X/M, PRS 1) and Doppler-mode (gain 61%, PRF 730 Hz, fre-  
915 quency 6.3 MHz, WF 4, PRS 3, PRC M/2) was used.

916 As previously reported by Pugliesi et al. [12], the CL was considered active when  
917 it presented luteal blood perfusion > 25%. In addition, after the ultrasonography, blood  
918 samples (25 mL) were collected from the jugular vein into sodium-heparinized tubes (BD  
919 Vacutainer; São Paulo; Brazil) for the isolation of immune cells (**Figure 1A**). Only ani-  
920 mals that showed active CL were submitted to blood collection.

921

922                    **3.2.3.1 Isolation of immune cells from peripheral blood**

923                    The PBMC and PMN were isolated by density gradient centrifugation using a  
924 Ficoll-Paque solution (GE Healthcare, Ref.17144003). For each cell isolation, whole  
925 blood was mixed with an equal volume of PBS in a 50-mL conical tube, and the solution  
926 was layered onto 15 mL Ficoll-Paque solution and centrifuged at 1100 g for 30 min at  
927 20°C. After centrifugation, the blood fractions are separated, as follows, plasma, buffy  
928 coat, and red blood cells together with PMN. The buffy coat was utilized for PBMC iso-  
929 lation, as described by Pugliesi et al. [12] and the last layer containing the granulocytes  
930 and red blood cells was utilized for PMN isolation, as described by Jiemtaweeboon et al.  
931 [21], with some modifications. The PBMC and PMN were subject to successive lyses  
932 steps with hypertonic solutions to lyse the red blood cells until a clean cell pellet was  
933 obtained. At the end of the isolation process, the cell pellet was re-suspended in medium  
934 according to its respective treatment. The purity of PBMC and PMN was checked by  
935 staining freshly isolated samples with the quick panoptic protocol. Samples were consid-  
936 ered pure when 95% of the 200 cells counted were mononuclear and polymorphonuclear  
937 cells. In addition, the viability was assessed pre- and post-culture with Trypan blue (0.4%,  
938 Sigma-Aldrich, Ref. T6146) reagent in a Neubauer camera, where samples that showed  
939 viability greater than 85% were considered viable (Supplementary Table 1).

940

941                    **3.2.3.2 Collection of UF on day 18 of the estrous cycle**

942                    After estrus, Holstein (*Bos taurus taurus*; N=10) non-lactating cows were sub-  
943 jected to AI with semen from a single sire (N=3) or remained as non-inseminated controls  
944 (N=3). On D18 post-estrus, all females were slaughtered and the reproductive tract (cer-  
945 vix, uterus, and ovaries) was collected, and immediately transported on ice to the labora-  
946 tory. The uterine horns of each reproductive tract were flushed simultaneously with 20

947 mL of buffer solution (PBS). When conceptus was present in the fluid, it was separated  
948 from the medium. At the end of the process, UF was centrifuged and the supernatant was  
949 removed and stored at -80°C for posteriorly use as a culture medium for immune cells.  
950 The fluid conditioned by the conceptus was denominated UF-Conceptus and the fluid  
951 from non-inseminated cows was used as a control (UF-Control). For use as a culture me-  
952 dium, a pool was made with the three fluid samples obtained from the UF-Conceptus  
953 group and the UF-Control group.

954

### 955 ***3.2.3.3 Experiment 1: Stimulation of immune cells with roIFNT***

956 Isolated PBMC (N=9;  $7 \times 10^6$  cells/mL) and PMN (N=10;  $5 \times 10^6$  cells/mL) were  
957 cultured in a 6-well plate (Kasvi, Ref. K12-006) in RPMI-1640 medium (Sigma-Aldrich;  
958 Ref. 22400071) containing 0.1% FBS (LGC; Ref. 10-bio-500) and Penicillin-Streptomy-  
959 cin (10  $\mu$ L/mL; Gibco™, Ref. 15140122) in combination with 100 ng/mL of roIFNT for  
960 24 or 3 h, respectively, in a humidified atmosphere at 37°C in 5% CO<sub>2</sub>. The dose of  
961 roIFNT used in the present study was determined based on studies obtained in the litera-  
962 ture [14, 22, 23], and validated in a pilot study using increasing doses of roIFNT (dose-  
963 response curve; 10, 100, or 1000 ng/mL). The PBMC and PMN without roIFNT treatment  
964 served as controls. After the incubation, the supernatant was removed and the collected  
965 cells were directed to RNA extraction and subsequent analysis of gene expression by  
966 qPCR.

967

### 968 ***3.2.3.4 Experiment 2: Culture of immune cells in UF***

969 Isolated PBMC (N=10;  $7 \times 10^6$  cells/mL) and PMN (N=8;  $5 \times 10^6$  cells/mL) were  
970 cultured in a 6-well plate (Kasvi, Ref. K12-006) in UF containing 0.1% FBS (LGC, Ref.  
971 10-bio-500) for 12 or 3 h, respectively, in a humidified atmosphere at 37°C in 5% CO<sub>2</sub>.

972 The PBMC and PMN cultivated in UF of non-pregnant cows (UF-Control) served as  
973 controls. After the incubation, the supernatant was removed and the collected cells were  
974 directed to RNA extraction and subsequent analysis of gene expression by qPCR.

975

### 976 **3.2.4 Experimental design of Experiment 3: accuracy of pregnancy markers** 977 **in PMN**

978 The PMN samples used in this present experiment were obtained from a previous  
979 study conducted by Dalmaso de Melo et al. [18], where, nulliparous (N=103), primipa-  
980 rous (N=53), and pluriparous (N=91) Nelore (*Bos taurus indicus*) cows were subjected to  
981 estradiol (E2) and P4 based protocol for synchronization of ovulation and TAI (TAI= day  
982 0 [D0]). On D20, the animals were evaluated for CL blood perfusion by color-Doppler  
983 ultrasound (MyLab Delta Vet Gold; Esaote Healthcare; Italy) and blood samples (25 mL)  
984 were collected from the jugular vein into sodium-heparinized tubes (BD Vacutainer; São  
985 Paulo; Brazil) for the isolation of PMN (**Figure 1B**).

986 PMN were isolated by Ficoll-Paque solution (GE Healthcare, Ref.17144003) gra-  
987 dient according to the methodology described in the *in vitro* study. After isolation, PMN  
988 were stored at -80°C for subsequent RNA extraction and gene expression analysis by  
989 qPCR. The purity of PMN was checked using the quick panoptic protocol according to  
990 the procedures described previously.

991

### 992 **3.2.5 RNA extraction, cDNA synthesis, and quantitative polymerase chain** 993 **reaction (qPCR)**

994 The isolated PBMC and PMN of the Experiments 1 and 2 were thawed on ice and  
995 the RNA was extracted using PureLink™ RNA Mini Kit (Invitrogen™, Ref.  
996 12183018A). Briefly, the PBMC and PMN pellets were dissolved using the lysis solution

997 and, in sequence, the RNA washing procedures were performed on the columns provided  
998 by the kit, as indicated in the manufacturer's instructions. For the Experiment 3, the iso-  
999 lated PMN was extracted by a modified protocol using Trizol™ (Thermo Fisher Scien-  
1000 tific, Ref. 15596018) reagent associated with the DirectZol-RNA kit (Zymo Research,  
1001 Ref. R2052), as described in detail by Dalmaso de Melo et al. [18].

1002 Total RNA concentration and purity were measured using a NanoVue™ Plus  
1003 spectrophotometer (GE Healthcare, UK), and samples with a 260/280 ratio ranging from  
1004 1.7 to 2.0 were used for transcript abundance analyses. The isolated RNA from samples  
1005 in both studies were treated with DNase I (DNase I Amplification Grade; Life Technolo-  
1006 gies, Ref. 18068015) to avoid genomic DNA contamination, as per the manufacturer's  
1007 instructions. Thus, the RNA isolated was subjected to reverse transcription using the  
1008 High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Ref. 4368814), ac-  
1009 cording to the manufacturer's instructions, and the cDNA of each sample was stored at -  
1010 20°C until qPCR analysis. Analyses of the relative abundance of transcripts were per-  
1011 formed using SYBR Green PCR Master Mix (Life Technologies, Ref. A25742) for am-  
1012 plification reactions in Step One Plus thermocycler (Applied Biosystems Real-Time PCR  
1013 System; Life Technologies, Ref. 4376600). The samples were run in triplicate and the  
1014 maximum CV accepted among the replicates was 0.1. Specific primers for each selected  
1015 gene (**Table 1**) were selected according to previous studies [12, 19, 23, 24, 25]. All de-  
1016 signed primers were evaluated for sequence specificity using BLAST  
1017 (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Furthermore, GeNorm software  
1018 (<https://genorm.cmgg.be>) was used to select reference genes, Glyceraldehyde-3-Phos-  
1019 phate Dehydrogenase (*GAPDH*) and Actin Beta (*ACTB*) were the most stable genes in  
1020 PMN and *GAPDH* and Ciclofilin (*PPIA*) were the most stable genes on PBMC. We used  
1021 LinRegPCR software to determine qPCR efficiency and quantification cycle (Cq) values

1022 per sample. Quantification was performed after normalization of the target gene expres-  
1023 sion values by the geometric mean of the endogenous control expression values, as de-  
1024 scribed by Pfaffl [26].

1025

### 1026 **3.2.6 Statistical analyses**

1027 The data were evaluated for detection of outliers using the Dixon test and the sig-  
1028 nificant ( $P < 0.05$ ) outliers detected were excluded from the analyses. The data that were  
1029 not normally distributed according to the Shapiro–Wilk test were transformed with nor-  
1030 mal logarithm, rank, and square root. Therefore, after applying data transformation when  
1031 necessary, they began to follow a normal distribution. The abundance of gene transcript  
1032 for all experiments was analyzed by analysis of variance (ANOVA) using the PROC  
1033 MIXED procedure of SAS (Version 9.2; SAS Institute). Pearson’s correlation between  
1034 ISG and cytokines expression was analyzed by the GraphPad Prism software (Version  
1035 5.0) for both studies. For the Experiment 1 and 2, animals were considered as a random  
1036 effect and the treatments (roIFNT or UF) as fixed effects in the model. Fold change was  
1037 calculated by the ratio between the gene expression of each sample in the treated group  
1038 (roIFNT or UF-Conceptus) and the average expression of the control group for each cell  
1039 type. In addition, the PROC POWER of SAS was used to determine the statistical power  
1040 for the comparisons of each end-point. For the Experiment 3, animals were considered as  
1041 a random effect, and fixed effects of group (pregnant or non-pregnant), category (nullip-  
1042 arous, primiparous, or pluriparous), and group-by-category interaction were included in  
1043 the model. The accuracy of the pregnancy diagnosis methods by ISG expression was cal-  
1044 culated by the number of false-negative (FN) and false-positive (FP) observations, nega-  
1045 tive predictive value (NPV), positive predictive value (PPV), specificity, and sensitivity,  
1046 as previously described by Pugliesi et al. [12]. A cutoff value for each ISG expression

1047 was determined through the establishment of a Receiving Operator Characteristic (ROC)  
1048 curve by using GraphPad Prism software. This software was also used to determine the  
1049 area under the curve (AUC) of each gene. The results are reported as mean  $\pm$  SEM. The  
1050 probability  $\leq 0.05$  indicated that the difference was significant and between  $0.05 > P \leq$   
1051  $0.10$ , indicating that the difference tended to be significant.

1052

### 1053 **3.3 RESULTS**

1054

#### 1055 **3.3.1 Experiment 1 and 2**

##### 1056 *3.3.1.1 Effects of roIFNT on the expression of ISG and cytokines in immune* 1057 *cells*

1058 To detect the magnitude response of peripheral blood PBMC and PMN by the  
1059 roIFNT, the specific immune-related genes including ISGs (*ISG15*, *RSAD2*, and *IFI44*),  
1060 pro-inflammatory (*IL1 $\beta$* ), and anti-inflammatory (*IL10*) cytokine were analyzed by  
1061 qPCR. In PBMC and PMN, the treatment with 100 ng/mL of roIFNT stimulated mRNA  
1062 expression of *ISG15*, *RSAD2*, and *IFI44* ( $P < 0.05$ ) compared to the Control group (no  
1063 further addition) (**Figure 2A, B**). When comparing the relative fold change of each gene  
1064 to the Control group, in PBMC treated with roIFNT, a greater ( $P < 0.0001$ ) stimulus was  
1065 observed in the *ISG15* and *RSAD2* genes than in the *IFI44* gene (**Figure 2C**). For PMN,  
1066 the relative fold change showed a greater ( $P = 0.05$ ) stimulus in the *RSAD2* gene com-  
1067 pared to other genes (**Figure 2D**).

1068 For the expression of cytokines in PBMC, expression of *IL1 $\beta$*  tended to be lesser  
1069 in the IFNT group ( $P = 0.10$ ); however, it was not enough to generate a significant re-  
1070 sponse in *IL10* expression ( $P=0.11$ ) (**Figure 3A**). In PMN, the expression of *IL1 $\beta$*   
1071 ( $P=0.15$ ) and *IL10* ( $P=0.85$ ) was not affected by roIFNT treatment (**Figure 3B**). When

1072 the relative fold change of the IFNT-treated group was compared to the Control group, a  
1073 greater fold change was detected for *IL10* (4.1-fold) compared to *IL1 $\beta$*  (0.7-fold) in  
1074 PBMC (P = 0.005) (**Figure 3C**). In PMN, the relative fold change did not differ signifi-  
1075 cantly (P = 0.14) between treatments (**Figure 3D**).

1076

### 1077 *3.3.1.2 Effects of UF from Day 18 on the expression of ISG and cytokines in* 1078 *immune cells*

1079 In this experiment, we compared whether UF from pregnant cows induced the  
1080 expression of ISG and immune genes in PBMC and PMN. The treatment with UF from  
1081 pregnant cows (UF-Conceptus) induced mRNA expression of *ISG15*, *RSAD2*, and *IFI44*  
1082 (P < 0.05) in PBMC and PMN (**Figure 4A, B**). When comparing the relative fold change,  
1083 a greater stimulus was observed in the *ISG15* and *RSAD2* genes than in the *IFI44* gene  
1084 for both, PBMC (P = 0.02) and PMN (P = 0.01) cultivated with UF-Conceptus compared  
1085 to UF-Control (**Figure 4C, D**).

1086 For the cytokines, a lesser expression of *IL1 $\beta$*  in PBMC (P = 0.007) and PMN (P  
1087 = 0.01) was detected in the UF-Conceptus group compared to the UF-Control (**Figure**  
1088 **5A, B**). However, no difference in *IL10* expression in PBMC (P = 0.14) and PMN (P =  
1089 0.44) was detected between the UF-Conceptus and UF-Control groups (**Figure 5A, B**).  
1090 When the relative fold change of the UF-Conceptus group was compared to the UF-Con-  
1091 trol group, a greater fold change was detected for *IL10* (2.3-fold) compared to *IL1 $\beta$*  (0.8-  
1092 fold) in PMN (P = 0.005) (**Figure 5D**). In PBMC, the relative fold change did not differ  
1093 significantly (P = 0.15) between treatments (**Figure 5C**).

1094

### 1095 *3.3.1.3 Correlations between ISG and cytokines in immune cells*

1096 For the Pearson's correlations determined in the PBMC treated with roIFNT or  
1097 UF (**Table 2**), six significant ( $P < 0.0001$ ) and strong ( $r > 0.8$ ) and two significant ( $P <$   
1098  $0.0001$ ) weak ( $r < 0.6$ ) correlations were detected. The strong positive correlations were  
1099 observed restrictedly between the ISGs (*ISG15 vs RSAD2*, *ISG15 vs IFI44*, and *RSAD2*  
1100 *vs IFI44*) in both stimuli (roIFNT and UF). For the comparison between ISGs vs cyto-  
1101 kines, two weak correlations were observed for *ISG15 vs IL10* for roIFNT treatment (pos-  
1102 itive correlation) and *IFI44 vs IL10* for UF treatment (negative correlation). There was  
1103 no other significant ( $P > 0.1$ ) correlation detected.

1104 In PMN treated with roIFNT or UF, the Pearson correlations (**Table 2**) determined  
1105 five significant ( $P < 0.0001$ ) strong ( $r > 0.8$ ) and two moderate ( $P = 0.01$ ) ( $0.6 < r < 0.8$ )  
1106 correlations. The strong positive correlations were observed restrictedly between the ISGs  
1107 for roIFNT treatment (*ISG15 vs RSAD2*, *ISG15 vs IFI44*, and *RSAD2 vs IFI44*) and UF  
1108 treatment (*ISG15 vs RSAD2* and *ISG15 vs IFI44*). For the comparison between ISGs vs  
1109 cytokines, moderate positive correlations were observed for *IL1 $\beta$  vs IL10* for roIFNT  
1110 treatment, and *RSAD2 vs IFI44* for UF treatment. There was no other significant ( $P > 0.1$ )  
1111 correlation detected.

1112

### 1113 **3.3.2 Experiment 3**

#### 1114 **3.3.2.1 Interferon stimulated genes – RSAD2 and IFI44**

1115 As demonstrated in **Figure 6**, the main effects of group, parity order category, and  
1116 the group-by-category interaction were significant for the *RSAD2* gene. The group-by-  
1117 category interaction represented that *RSAD2* abundance did not differ among the parity  
1118 order categories in the pregnant females; whereas, in non-pregnant females, the relative  
1119 expression of *RSAD2* was lower in pluriparous compared to nulliparous and primiparous  
1120 females. Also, the abundance was 3.2, 4.4, and 8.5-fold greater ( $P < 0.007$ ) in the pregnant

1121 than non-pregnant nulliparous, primiparous and pluriparous females, respectively. For  
1122 *IFI44*, the main effects of group and parity order category were significant (**Figure 6**).  
1123 The *IFI44* abundance was 3.8-fold greater in the pregnant group compared to the non-  
1124 pregnant ( $P < 0.0001$ ). A parity category effect ( $P < 0.0001$ ) indicated a higher expression  
1125 of *IFI44* in nulliparous, followed by a lower expression in pluriparous and primiparous  
1126 cows.

1127

### 1128 **3.3.2.2 Correlations between ISG in PMN**

1129 As demonstrated in **Table 3**, Pearson's correlations determined between the ISGs  
1130 indicated three strong ( $r > 0.8$ ), five moderate ( $0.6 < r < 0.8$ ), and six weak ( $r < 0.6$ )  
1131 positive correlations in the PMN on day 20 post-TAI. In nulliparous, one strong (*ISG15*  
1132 *vs OAS1*), three moderate (*ISG15 vs RSAD2*, *OAS1 vs RSAD2*, and *RSAD2 vs IFI44*), and  
1133 two weak (*ISG15 vs IFI44* and *OAS1 vs IFI44*) correlations were observed. In primipa-  
1134 rous and pluriparous cows, the correlations were similar, with one strong (*ISG15 vs*  
1135 *OAS1*), one moderate (*RSAD2 vs IFI44*), and two weak (*ISG15 vs IFI44* and *OAS1 vs*  
1136 *IFI44*) correlations. There was no other significant ( $P > 0.10$ ) correlation detected.

1137

### 1138 **3.3.2.3 Accuracy of pregnancy predictors**

1139 The cutoff values for ISG genes (*RSAD2*, *IFI44*, *ISG15*, and *OAS1*) were estab-  
1140 lished through ROC curve analysis (**Figure 7**). A different cutoff value was established  
1141 for nulliparous (*RSAD2*=0.92, *IFI44*=0.0086, *ISG15*=1.27 and *OAS1*=0.53), primiparous  
1142 (*RSAD2*=0.48, *IFI44*=0.0048, *ISG15*=1.04 and *OAS1*=0.48) and pluriparous cows  
1143 (*RSAD2*=0.79, *IFI44*=0.0058, *ISG15*=0.31 and *OAS1*=0.53). As demonstrated in **Table**  
1144 **4**, in nulliparous heifers, the accuracy for the *IFI44* gene was better when compared to  
1145 the *RSAD2* gene (86% *vs* 79%, respectively). In this case, the expression of *IFI44* had a

1146 lower number of FN (7/100) and, consequently, a higher NPV (86.3%) and sensitivity  
1147 (85.7%) when compared to the expression of *RSAD2* (10/100). On the other hand, in pri-  
1148 miparous females, the accuracy was better for the *RSAD2* when compared to the *IFI44*  
1149 gene (90% vs 84%). The expression of *RSAD2* in this category showed a lower number  
1150 of FN (0/50) and, consequently, a higher NPV (100%) and sensitivity (100%) when com-  
1151 pared to the *IFI44* (4/50). In addition, in pluriparous cows, the accuracy was similar be-  
1152 tween both ISGs (*RSAD2*=92.8% vs *IFI44*=91.6%), as well as, the number of FN, NPV,  
1153 and sensitivity (**Table 4**).

1154         Considering the absence of FN results when using Doppler ultrasonography [12,  
1155 17, 18], a combined method between Doppler-US and ISG was approached, characterized  
1156 by the use of the ISG method only in females with a functional CL (blood perfusion >  
1157 25%) on D20. Thus, as demonstrated in **Table 5**, in nulliparous females, the accuracy  
1158 was better and similar using the combination of two (*RSAD2/IFI44*, accuracy: 90%) or  
1159 four (*RSAD2/IFI44/ISG15/OAS1*, accuracy: 91%) ISGs. However, the combination of  
1160 four ISGs had a lower number of FN (1/100), and consequently, greater NPV (97.7%)  
1161 and sensitivity (98%) when compared to the combination of two ISGs. In primiparous  
1162 cows, the accuracy was similar and better when using only the *RSAD2* gene (accuracy:  
1163 98%) or using the combination of two ISGs (*RSAD2/IFI44*, accuracy: 98%). In pluripar-  
1164 ous cows, the greatest accuracy was obtained using two ISGs (*RSAD2/IFI44*, accuracy:  
1165 94%); however, the combination of four ISGs had the lowest number of FN (1/83), and  
1166 consequently, greater NPV (96.5%) and sensitivity (98%).

1167         In **Table 6**, the same method of the ISG was used only in females with a functional  
1168 CL on D20 regardless of the parity category. The accuracy was similar between all ISG  
1169 combinations. However, the combination of four ISGs (*RSAD2/IFI44/ISG15/OAS1*) had

1170 a lower number of FN (2/233), and consequently, greater NPV (97.9%) and sensitivity  
1171 (98.4%), compared to other combinations.

1172

### 1173 **3.4 DISCUSSION**

1174 The endocrine actions of IFN- $\tau$  have been proposed to affect several cells and  
1175 tissues, such as the peripheral immune cells through ISGs [12, 23]. Evaluation of ISG  
1176 transcript abundance in circulating immune cells has been investigated as a marker for  
1177 pregnancy diagnosis purposes in ruminants, as it indirectly signals the presence of the  
1178 conceptus [12, 27, 28, 29]. Thus, the development of an applied method using ISG abun-  
1179 dance to detect early pregnancy or predict embryonic/fetal mortality could contribute to  
1180 the improvement of bovine production systems, reducing the time from AI to resynchro-  
1181 nization and rebreeding. In the herein study, we compared for the first time the relative  
1182 expression of ISGs in PBMC and PMN in front of direct IFN- $\tau$  stimulus or stimulated by  
1183 a medium conditioned by the conceptus on day 18 of pregnancy. The original results  
1184 indicate the non-classic ISG, *RSAD2* as the most stimulated biomarker of bovine concep-  
1185 tus signaling in PBMC and PMN. In addition, the accuracy of these ISGs as predictors of  
1186 early pregnancy in PMN on day 20 post-TAI in bovine females was determined. Thus,  
1187 the novel results demonstrated that the association of classic (*ISG15* and *OAS1*) and non-  
1188 classic (*RSAD2* and *IFI44*) ISGs with the color-Doppler diagnosis is an advanced method  
1189 to differentiate with high accuracy pregnant and non-pregnant *Bos indicus* beef heifers  
1190 and cows on day 20 post-TAI.

1191 Initially, we characterized the magnitude response of ISGs in PBMC and PMN  
1192 when stimulated *in vitro* with 100 ng/mL rIFNT or UF from pregnant cows. To the best  
1193 of our knowledge, the use of a conceptus-conditioned medium on day 18 of pregnancy  
1194 has never been attempted previously to investigate the physiological stimulus generated

1195 by the conceptus on immune cells. Based on the recent results of our group suggesting  
1196 new candidates for pregnancy prediction based on a transcriptome analysis in PBMC and  
1197 PMN on day 18 of pregnancy [19], the use of these biomarkers may be more accurate in  
1198 predicting early pregnancy when compared to classic ISGs [12, 18]. Therefore, we se-  
1199 lected one classic (*ISG15*) and two non-classic (*RSAD2* and *IFI44*) ISGs identified by the  
1200 transcriptome analysis to evaluate the transcript abundance of mRNA, and subsequent  
1201 sensitivity and specificity analysis as a potential early pregnancy marker. For both types  
1202 of immune cells, the treatments with 100 ng/mL of roIFNT or UF from pregnant cows  
1203 were able to stimulate the expression of all ISGs (*ISG15*, *RSAD2*, and *IFI44*) evaluated.  
1204 This result is due to the direct and rapidly effect of exogenous addition of IFN- $\tau$  or by its  
1205 presence in the UF from pregnant cows. Also, the fold change analysis showed that *ISG15*  
1206 and *RSAD2* were the most stimulated ISG in PBMC and PMN under the *in vitro* condi-  
1207 tions. In this context, Shirasuna et al. [23] reported that recombinant bovine IFN- $\tau$   
1208 (rbIFNT) treatment (0.1-10 ng/mL) regulated the mRNA expression of classic ISGs  
1209 (*ISG15* and *OAS1*) in PBMC and PMN cultured *in vitro* for 24 h. Similarly, Forde et al.  
1210 [14] found that the expression of non-classic ISGs (*IFIT2*, *SAMD9*, and *USP18*) was di-  
1211 rectly induced by IFN- $\tau$  in bovine endometrial cells treated *in vitro* with 100 ng/mL or 1  
1212  $\mu$ g/mL of roIFNT for 30 min, 2 h or 24 h. In addition, Rashid et al. [30] reported upreg-  
1213 ulation of *ISG15* and *OAS1* in PBMCs cultured with UF from day 7 of pregnant cows.  
1214 These results suggest that the stimulation of ISGs in peripheral blood immune cells due  
1215 to the presence of a viable conceptus and/or IFN- $\tau$  is essential for the establishment of  
1216 pregnancy. Thus, it generates the possibility of developing reliable indices of earlier preg-  
1217 nancy than other existing methods in cattle.

1218         During early pregnancy establishment, a delicate harmony between pro- and anti-

1219 inflammatory cytokines is needed to increase the maternal tolerance towards the semi-  
1220 allogeneic embryo [31]. Thus, any disturbance in the Th1:Th2 cytokine balance may lead  
1221 to embryonic/fetal mortality and failure in pregnancy [32]. Here, we investigated the re-  
1222 sponse of pro (*IL1 $\beta$* ) and anti-inflammatory (*IL10*) cytokines to stimulation of roIFNT or  
1223 conceptus-conditioned medium. Surprisingly, UF from pregnant cows only suppressed  
1224 the expression of the *IL1 $\beta$*  cytokine in PBMC and PMN. Similarly, there was a tendency  
1225 to reduce *IL1 $\beta$*  cytokine transcripts induced by roIFNT treatment in PBMC. Although the  
1226 difference did not approach significance ( $P=0.11$ ), the *IL10* abundance in PBMC treated  
1227 with roIFNT follows the same direction of upregulation of anti-inflammatory cytokines  
1228 reported in earlier studies [23, 30, 33]. A significant difference between pregnant statuses  
1229 was not observed probably due to the high variation within each experimental group, as  
1230 our statistical power obtained for this end-point was moderate to high (from 0.49 to 0.89).  
1231 In addition, the analysis of fold change indicated a significant difference in between the  
1232 abundance of *IL10* (upregulated) and *IL1 $\beta$*  (downregulated) in both immune cells and  
1233 stimuli. The findings of the herein study corroborate with Rashid et al. [30], who verified  
1234 downregulation of pro-inflammatory cytokines (*IL1 $\beta$*  and *TNF $\alpha$* ) and upregulation of  
1235 anti-inflammatory cytokines (*IL10* and *TGF $\beta$ 1*) in PBMCs and bovine uterine epithelial  
1236 cells stimulated with UF from day 7 of pregnant cows or rbIFNT for 12 and 24 h, respec-  
1237 tively. Likewise, when PMNs were stimulated with 10 ng/mL of rbIFNT for 3 h, they  
1238 generated an anti-inflammatory response through upregulation of *TGF $\beta$*  and downregu-  
1239 lation of the pro-inflammatory cytokine *TNF $\alpha$*  [33]. In this context, the analysis of tran-  
1240 scripts of pro- and anti-inflammatory cytokines suggests that the environment condi-  
1241 tioned by the conceptus modulates the uterine immunological environment to accept the  
1242 semi-allogeneic embryo and induce a state of immunological tolerance through the sup-

1243 pression of *IL1 $\beta$* , essential for the survival of the embryo and the establishment of preg-  
1244 nancy. Furthermore, we believe that possibly other molecules present in the UF from day  
1245 18 of pregnancy cows are acting in synergy with IFN- $\tau$  to induce an anti-inflammatory  
1246 state, observed in immune cells.

1247         Based on studies obtained in the literature, there are differences in the concentra-  
1248 tion of components found in the UF of pregnant and non-pregnant cows. In a study con-  
1249 ducted by Sponchiado et al. [34], the results demonstrate that the pre-hatched embryo  
1250 changes the uterine microenvironment as early as the 7th day after estrus *in vivo*. Such  
1251 modulation included changes in the concentrations of metabolites derived from lipoxy-  
1252 genase, amino acids, biogenic amines, acylcarnitines, and phospholipids measured in the  
1253 UF of pregnant and non-pregnant cows. Likewise, proteomic analysis of UF on days 16  
1254 and 18 of pregnancy indicated nine proteins upregulated in the UF of pregnant cows com-  
1255 pared to the non-pregnant cows [35]. The abundant proteins in pregnancy included five  
1256 enzymes involved in biosynthetic pathways (carbonic anhydrase, isocitrate dehydrogen-  
1257 ase, NDPK, PNP, and triosephosphate isomerase) which probably reflects the increased  
1258 metabolic activity of the endometrium during pregnancy. Isocitrate dehydrogenase was  
1259 previously found in greater abundance on day 18 of gestation compared to non-pregnant  
1260 bovine endometrial tissue [36]. Still, two others are antioxidant enzymes (peroxiredoxin  
1261 1 and thioredoxin) that possibly protect the trophoblast cells of the developing conceptus  
1262 against oxidative stress during their rapid cell division [37].

1263         The present research provides evidence that the expression of ISGs in both PMNs  
1264 and PBMCs could be used as a potential tool for early detection of pregnant bovine fe-  
1265 males. The period between days 18 and 20 of pregnancy was classified as the most ap-  
1266 propriate for carrying out pregnancy diagnosis using ISG expression in immune cells in  
1267 previous studies, as this period was associated with less data overlap between pregnant

1268 and non-pregnant animals. The lower number of overlaps at this time point results from  
1269 the greater secretion of IFN- $\tau$  around the 20th day of gestation as IFN- $\tau$  increases propor-  
1270 tionally to the growth of the trophoctoderm [38, 39]. Previous studies have suggested that  
1271 the PMN may have an earlier response to IFN- $\tau$  secretion. Toji et al. [40] indicated that  
1272 PMNs are more sensitive to IFN- $\tau$  and that the shorter lifespan (a few hours) of neutro-  
1273 phils may impact PMN sensitivity to IFN- $\tau$  compared to other immune cell types. Thus,  
1274 based on the results obtained in the *in vitro* studies, we aimed to evaluate whether the  
1275 non-classic ISGs (*RSAD2* and *IFI44*) can be accurate predictors for early diagnosis of  
1276 pregnancy in PMN on D20 post-TAI through an *in vivo* study. To our knowledge, the use  
1277 of a single assessment of non-classic ISG expression and its accuracy using a large num-  
1278 ber of animals has never been explored as a tool to prospectively distinguish pregnant and  
1279 non-pregnant females. The PMN samples used in this study were obtained in a prospec-  
1280 tive study conducted by Dalmaso de Melo et al. [18], where the accuracy of a pregnancy  
1281 detection method was evaluated through the abundance of classic ISGs (*ISG15* and  
1282 *OAS1*). The herein results expanded the evaluation of PMNs on D20 indicating a higher  
1283 abundance of both non-classic ISGs tested (*RSAD2* and *IFI44*) in pregnant compared to  
1284 non-pregnant females. These results are consistent with the expression of classic ISGs in  
1285 PBMC and total blood immune cells reported in beef cows [12, 27] and dairy heifers and  
1286 cows [28, 41, 42].

1287         Furthermore, in non-pregnant females, the abundance of *RSAD2* was higher in  
1288 nulliparous and primiparous than in pluriparous females. For *IFI44*, transcript abundance  
1289 was higher in nulliparous, followed by pluriparous and primiparous cows, regardless of  
1290 gestational status. The reason for the difference in the ISG response among the categories  
1291 of parity is unspecified. One possible explanation is that the size of the embryo is different  
1292 in nulliparous and pluriparous cows and this difference in size affects the total capacity

1293 of IFN- $\tau$  production by the embryo. In this regard, Berg et al. [43] reported that heifers  
1294 have larger embryos compared to cows during the MRP period. Thus, possibly this dif-  
1295 ference persists for younger (primiparous) cows compared to older (pluriparous) cows.  
1296 In addition, there could also be differences in immune function for nulliparous and plu-  
1297 riparous cows that predispose pluriparous cows to a lesser IFN- $\tau$ -stimulated response,  
1298 since pluriparous cows have already had previous contact with IFN- $\tau$  in previous preg-  
1299 nancies. Another possibility is that the overall size of pluriparous cows is larger compared  
1300 to younger animals, and this may create a dilution effect that reduces IFN- $\tau$  concentra-  
1301 tions and the ISG response in blood leukocytes [41]. Furthermore, there are differences  
1302 in overall body metabolism between different parity order categories, which could affect  
1303 IFN- $\tau$  concentrations. For example, in primiparous cows, there is a greater energy demand  
1304 compared to pluriparous cows and heifers, since first parous animals need to distribute  
1305 their energy for lactation and growth [44]. However, the findings of the herein study are  
1306 adverse, since, the effects of parity order did not follow the same pattern in *RSAD2* and  
1307 *IFI44* genes. In this regard, previous studies report that the expression of the *RSAD2* gene  
1308 in the ovine uterus [45], and in PBMCs of beef cows [46] are regulated by P4 concentra-  
1309 tions; however, whether this regulation is positive or negative remains contradictory be-  
1310 tween studies. In this context and based on the results of the herein study considering the  
1311 effect of parity order category on the *RSAD2* gene, we speculate that the lower expression  
1312 of this gene in primiparous cows can be explained by the lower circulating P4 concentra-  
1313 tions. Since, in first parous cows reduced circulating P4 is expected due to the ovulatory  
1314 follicle, and subsequent developed CL, which are smaller in size compared to pluriparous  
1315 cows. Furthermore, primiparous cows exhibit greater metabolism of steroid hormones  
1316 compared to nulliparous and pluriparous females [44]. Thus, the combination of these  
1317 factors can lead to a lower concentration of this hormone, influencing *RSAD2* expression.

1318           Based on previous studies [12, 18, 47] the ROC curve was established to deter-  
1319 mine the efficiency of pregnancy predictors through the expression of the classic ISGs  
1320 (*ISG15* and *OASI*) obtained in the study by Dalmaso de Melo et al. [18]; and non-classic  
1321 ISGs (*RSAD2* and *IFI44*) obtained in the herein study. The ROC curve showed that all  
1322 ISGs were considered significant predictors of pregnancy, and in primiparous and plurip-  
1323 arous cows, *RSAD2* and *IFI44* were considered the most accurate genes. When ISG ex-  
1324 pression was compared between parity categories, greater and similar accuracies were  
1325 observed, respectively, in primiparous and pluriparous compared to nulliparous females.  
1326 These findings are contrary to those observed by Dalmaso de Melo et al. [18], where the  
1327 accuracy of the *ISG15* and *OASI* genes was higher in heifers (81%) compared to cows  
1328 (72%). Pugliesi et al. [12] described an accuracy close to 80% when this method was  
1329 performed on PBMCs on the 20th day of pregnancy in beef cows, but sensitivity ranged  
1330 from 66 to 78%. Yoshino et al. [47] reported for ISG expression PMN of dairy cows,  
1331 accuracies ranging from 57 to 86% between days 20 and 22 of gestation. Thus, we could  
1332 obtain better accuracies in the present study, as the accuracy of *RSAD2* and *IFI44* was  
1333 higher due to the lower number of FP and FN, compared to previous studies that used  
1334 only classic ISGs. But, even with better accuracy, the FP and FN results were not miti-  
1335 gated using these novel biomarkers, and this impacts negatively for use of this method  
1336 commercially on the field.

1337           Thus, considering the increasing use of the color-Doppler methodology in com-  
1338 mercial beef cattle operations [48] for detecting non-pregnant females with high efficacy  
1339 (100% sensitivity), and as its main limitation is the number of FP results, we combined  
1340 this method with the expression of ISGs in PMN. For this, the cutoff value of each gene  
1341 individually or together (*RSAD2*, *IFI44*, *RSAD2/IFI44*, or *RSAD2/IFI44/ISG15/OASI*)  
1342 was applied only in females with a functional CL on D20; while females with a non-

1343 functional CL on D20 were automatically classified as non-pregnant. Overall, the com-  
1344 bined method increased the accuracy of the diagnosis due to the reduction in FP results,  
1345 but the FN results were still frequent. Similarly, Pugliesi et al. [12] and Dalmaso de Melo  
1346 et al. [18] reported a better accuracy between 84 to 90% when combining the use of two  
1347 genes (*OAS1/MX2* or *ISG15/OAS1*) in females with a functional CL on day 20 of preg-  
1348 nancy.

1349 In a final approach, the expression of ISGs in animals with active CL on D20  
1350 regardless of the parity category was evaluated, and all possible combinations between  
1351 genes were performed. In this circumstance, all combinations presented satisfactory pre-  
1352 cision, and the combination between the four ISGs (*RSAD2/IFI44/ISG15/OAS1*) pre-  
1353 sented the lowest proportion of FN (0.9%; 2/233). Therefore, even combining the ISG  
1354 expression + color-Doppler methods, the FP and FN results were not eradicated, which  
1355 in practical situations could cause financial losses, mainly due to the FN results. The in-  
1356 accuracy for predicting pregnancy status associated with FN results may be related to  
1357 animals in which IFN- $\tau$  does not signal enough to stimulate the expression of ISGs. In  
1358 this context, it is known that the size of the conceptus is directly correlated with the  
1359 amount of IFN- $\tau$  released [49]. On the other hand, the inaccuracy related to FP results can  
1360 occur as an outcome of early embryonic mortality or the induction of ISGs by other types  
1361 of stimuli. Interferon receptors (IFNAR) are non-selective receptors and can be stimu-  
1362 lated by any type 1 interferon, which means that other interferons can bind to it and stim-  
1363 ulate the expression of ISGs, as, for example, in viral infections [12, 18, 42, 50]. Thus,  
1364 progress in methodology are needed so that more accurate and easy-to-apply methods can  
1365 be developed and routinely used in the reproductive management of beef and dairy fe-  
1366 males.

1367           In summary, we conclude that immune cells respond promptly to IFN- $\tau$  and/or  
1368 conceptus stimulus, which may favor the use of PBMC or PMN in novel methods for the  
1369 detection of pregnancy in cattle. Furthermore, the presence of the bovine conceptus in the  
1370 uterine environment induces a state of maternal immune tolerance essential for embryonic  
1371 survival and the establishment of pregnancy. The greater abundance of *RSAD2* and *IFI44*  
1372 in PMN on day 20 post-TAI in pregnant beef heifers and suckled cows allows a high-  
1373 accuracy method to detect pregnancy, but FN results are not eradicated. In addition, for  
1374 the first time, our study reports that the association between the expression of classic and  
1375 non-classic ISGs can be used to obtain a more accurate method of pregnancy prediction  
1376 in bovine females with function CL at early pregnancy.

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1589           **3.6 FINAL CONSIDERATIONS**

1590           The development of methods for early detection of pregnancy or embryonic/fetal  
1591 mortality can contribute to improving the efficiency of reproductive programs in cattle  
1592 worldwide. In this context, modulating the maternal immune system during early preg-  
1593 nancy has been reported as one of the main factors for gestational success and a signifi-  
1594 cant cause of embryonic loss.

1595           In the first studies (*Experiments 1 and 2*) described in Chapter 2, we initially  
1596 sought to understand the response of immune cells from *Bos taurus indicus* heifers, stim-  
1597 ulated by treatments with exogenous IFN- $\tau$ , or conditioned medium by the conceptus,  
1598 through an *in vitro* cell culture system. In this model, we identified that the presence of a  
1599 viable bovine conceptus modulates maternal immunological patterns in the uterus in favor  
1600 of a TH2 anti-inflammatory response, through the downregulation of the cytokine *IL1 $\beta$* ,  
1601 favoring the establishment of pregnancy. However, further studies are needed to elucidate  
1602 which molecules secreted or induced by the conceptus, in addition to IFN- $\tau$ , may be in-  
1603 ducing these maternal immune responses. Furthermore, we also observed that both treat-  
1604 ments induced the expression of classic and novel ISGs, with *ISG15* and *RSAD2* being  
1605 the most stimulated genes in both cell types, indicating that these markers have the po-  
1606 tential to be evaluated for their accuracy as early pregnancy predictors on day 20 post-  
1607 TAI. These findings may influence the improvement of molecular-based tests using ISGs  
1608 to detect pregnancy in cattle, in addition to generating improvements in production sys-  
1609 tems, especially those that use fixed-time programs.

1610           Although several reports describe a more pronounced response of PMNs to stim-  
1611 ulation with IFN- $\tau$ , in the present study, we found no significant difference in the expres-  
1612 sion of ISGs between cell groups. Therefore, the response of PMNs was similar to that  
1613 described in PBMCs, leading us to believe that both groups of cells can be used with

1614 similar efficacy for the diagnosis of pregnancy. Therefore, based on the results obtained  
1615 in the *in vitro* studies, we conducted an accuracy experiment (*Experiment 3*) using only  
1616 PMNs.

1617 To better select circulating transcripts for use as potential markers, larger numbers  
1618 of animals under field conditions are necessary. For this reason, we conducted an *in vivo*  
1619 study, where we analyzed the accuracy of the ISGs selected in the *Experiments 1 and 2*,  
1620 as early pregnancy predictors in bovine females of different parity categories. Although  
1621 all ISGs evaluated were classified as accurate predictors of pregnancy on D20 post-TAI,  
1622 the use of these genes for pregnancy diagnosis still needs to overcome some barriers for  
1623 practical application in the area. Firstly, although the expression of ISGs when associated  
1624 with Doppler-US has generated satisfactory accuracy, the considerable number of false  
1625 positive results, and mainly false negatives, significantly reduces the precision of the  
1626 technique. Secondly, the technique for isolating immune cells and subsequent analysis of  
1627 transcripts by qPCR is expensive and time-consuming, which would not justify the choice  
1628 of this technique over others, using current methodologies. Furthermore, it is known that  
1629 there is a difference in the expression patterns of ISGs between younger animals (heifers)  
1630 compared to older (cows), making the use of this method even more limited.

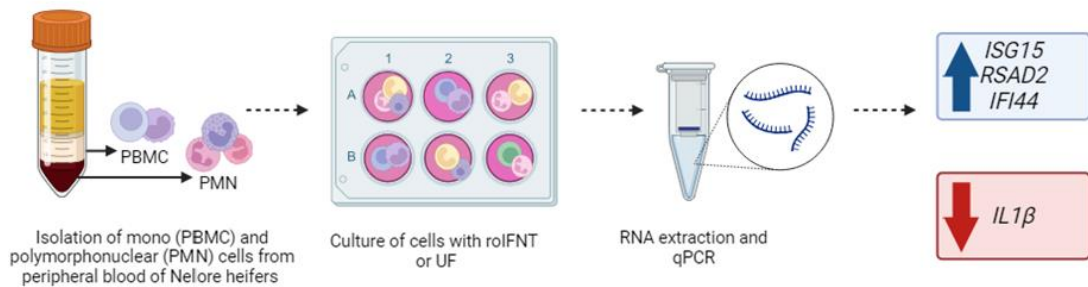
1631 Despite the obstacles raised, we believe that research using ISGs as pregnancy  
1632 markers should be encouraged, as these genes respond early to a direct stimulus from the  
1633 conceptus during early pregnancy. Furthermore, technology is constantly evolving, and  
1634 today more sensitive and faster techniques are available, such as gene expression analysis  
1635 by digital PCR, and more efficient forms of cell isolation. Moreover, the abundance of  
1636 ISGs in the whole blood fraction could be explored, as we conclude that the cellular frac-  
1637 tion does not affect the expression of these genes. This opens the way for the development

1638 of rapid commercial kits, as well as metabolite analysis techniques through metabolom-  
1639 ics, which can facilitate the application of this technique in the field.

1640

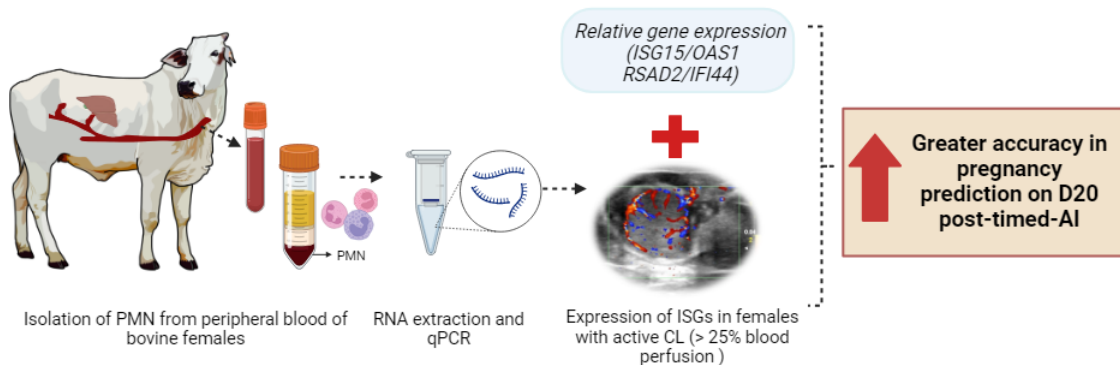
1641 **3.7 FIGURES AND LEGENDS**

***In vitro* study: stimulation of immune cells with recombinant ovine IFN- $\tau$  (roIFNT) or uterine flush (UF)**



1642

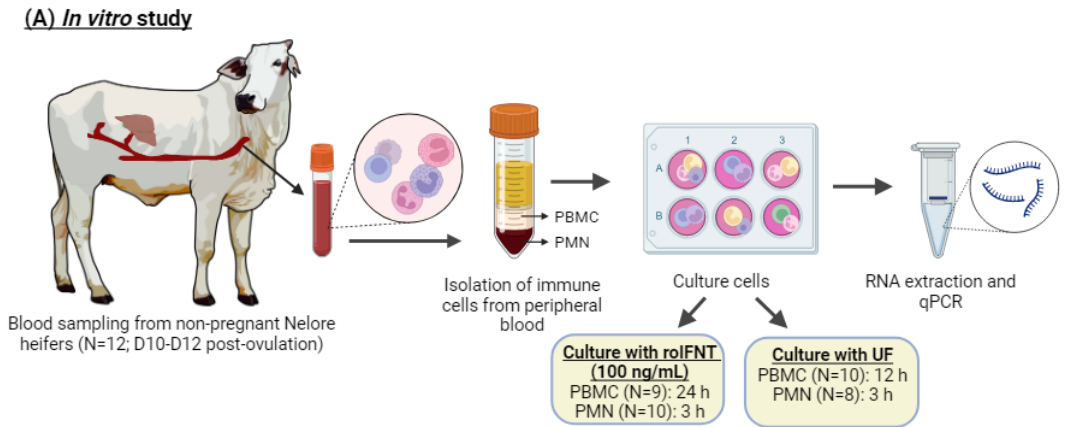
***In vivo* study: accuracy of pregnancy markers in PMN cells**



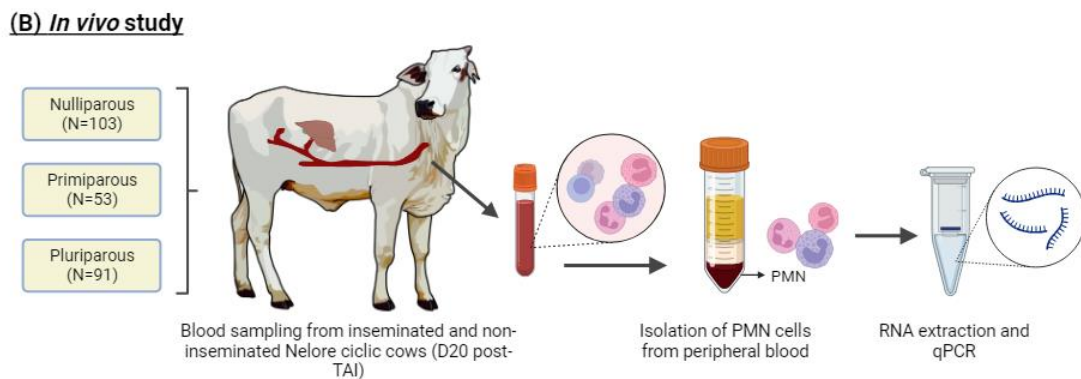
1643

1644 **Graphical abstract.** In the *in vitro* studies (**Experiments 1 and 2**), relative expression indicated  
1645 that the treatments upregulated the ISGs (*ISG15*, *RSAD2*, and *IFI44*) and downregulated the pro-  
1646 inflammatory cytokine *IL1 $\beta$*  in PBMC and PMN immune cells. According to the *in vivo* study  
1647 (**Experiment 3**), we propose that an association between the expression of classic (*ISG15* and  
1648 *OAS1*) and non-classic (*RSAD2* and *IFI44*) ISGs in females with active CL through Doppler ul-  
1649 trasonography can be used as a high-accurate predictor of pregnancy in cows on day 20 post-  
1650 timed-AI. Blue arrows represent downregulation and red arrows represent upregulation of genes.

1651



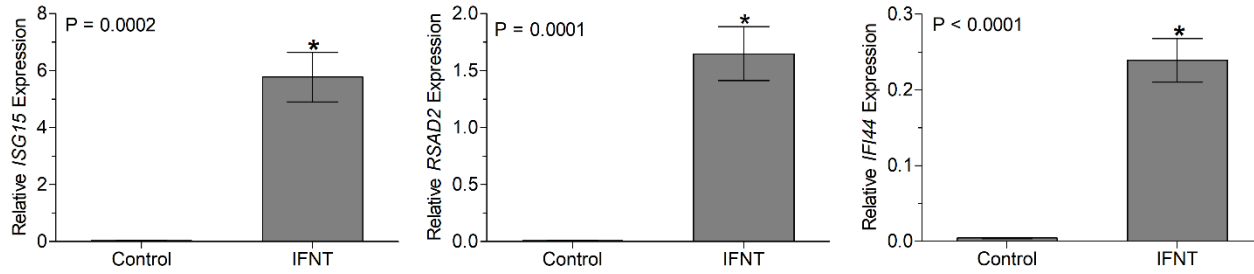
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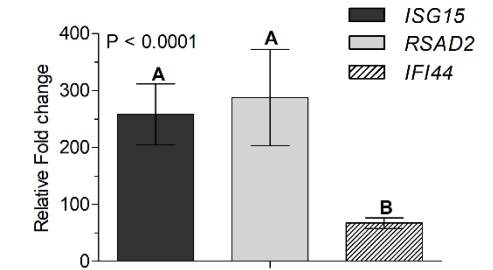
1653

1654 **Figure 1.** Schematic representation of the experimental design. (A) In the *in vitro* studies, non-  
 1655 pregnant Nelore heifers (N=12) were submitted to blood sampling collection between D10-D12  
 1656 post-ovulation (D0=day of ovulation), for the isolation of mononuclear (PBMC) and polymor-  
 1657 phonuclear (PMN) cells. Isolated PBMC and PMN were stimulated with 100 ng/mL roIFNT  
 1658 (IFNT group, **Experiment 1**) or uterine flush from day 18 pregnant cows (UF-Conceptus, **Exper-**  
 1659 **iment 2**) for 24 h (PBMC) or 3 h (PMN) at 37°C in 5% CO<sub>2</sub>. The groups without treatment [Con-  
 1660 trol or UF from cows on day 18 of the diestrus phase (UF-Control)] served as controls. After the  
 1661 incubation, the cells were directed to RNA extraction and gene expression was determined by  
 1662 qPCR. (B) For the *in vivo* study (**Experiment 3**), Nelore females (nulliparous, N=103; primipa-  
 1663 rous, N=53; pluriparous, N=91) were submitted to timed-AI (TAI) on day 0. On D20 post-TAI,  
 1664 PMN was isolated from the peripheral blood of inseminated and non-inseminated cows. After  
 1665 isolation, PMN was directed to RNA extraction, and gene expression was determined by qPCR  
 1666 for the accuracy of pregnancy predictors.

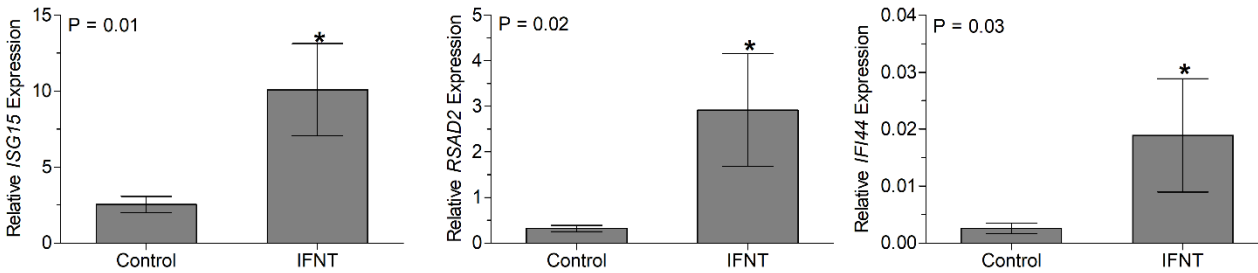
**(A) PBMC**



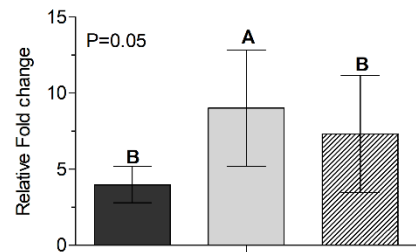
**(C) PBMC**



**(B) PMN**



**(D) PMN**



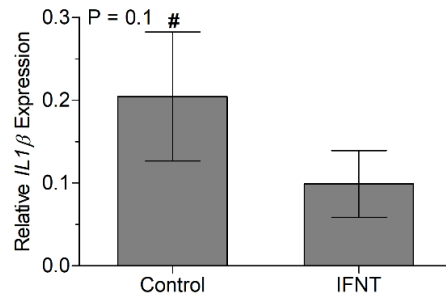
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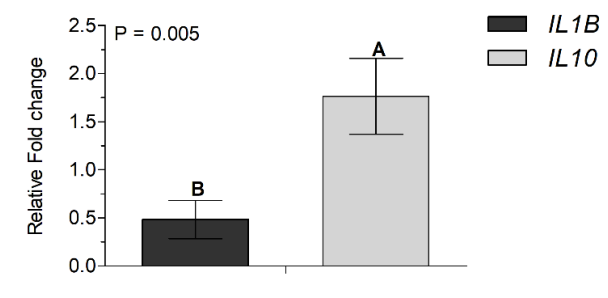
1669 **Figure 2.** Mean  $\pm$  SEM for relative expression and fold change of *ISG15*, *RSAD2*, and *IFI44* genes by qPCR in (Panel A, C) PBMC (N=9) cultured for 24 h and  
1670 (Panel B, D) PMN (N=10) cultured for 3 h, and treated (100 ng/mL roIFNT) or untreated (Control) with recombinant ovine interferon- $\tau$  (roIFNT). \*<sup>AB</sup> An  
1671 asterisk or different letters above the bar indicates a significant difference ( $P \leq 0.05$ ) between the transcripts.

1672

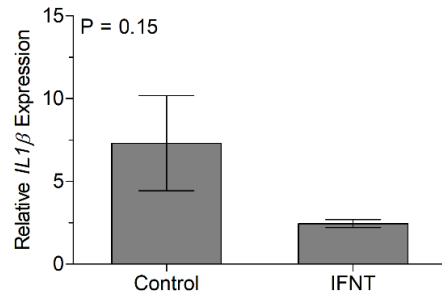
**(A) PBMC**



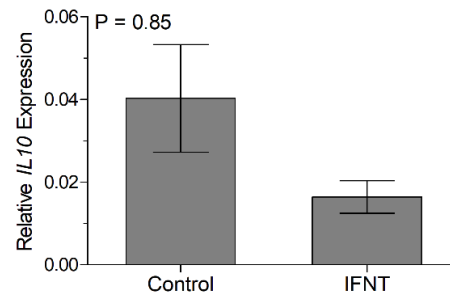
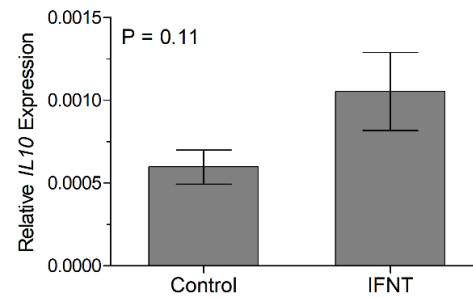
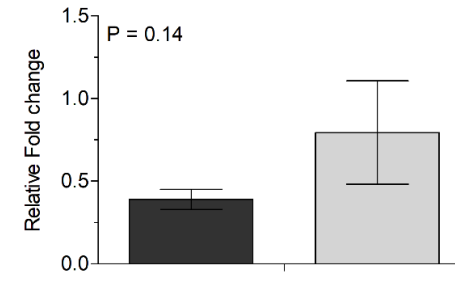
**(C) PBMC**



**(B) PMN**



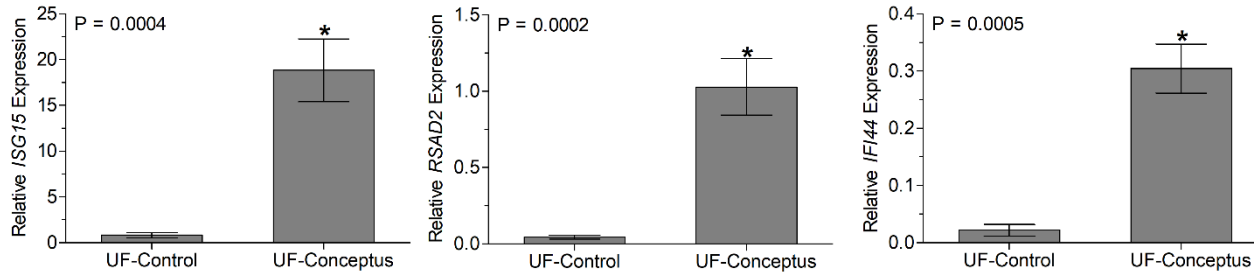
**(D) PMN**



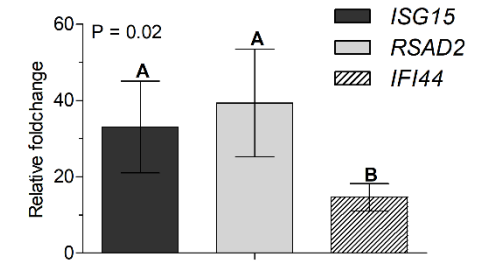
1673

1674 **Figure 3.** Mean  $\pm$  SEM for relative expression and fold change of pro-inflammatory (*IL1β*) and anti-inflammatory (*IL10*) cytokine genes by qPCR in (Panel A,  
1675 C) PBMC (N=9) cultured for 24 hours and (Panel B, D) PMN (N=10) cultured 3 hours, and treated (100 ng/mL roIFNT) or untreated (Control) with recombinant  
1676 ovine interferon-tau (roIFNT). #A hatch tag above the bar indicates a tendency to significance ( $0.05 < P \leq 0.1$ ) between the transcripts. <sup>AB</sup> Different letters above  
1677 the bar indicates a significant difference ( $P \leq 0.05$ ) between the transcripts

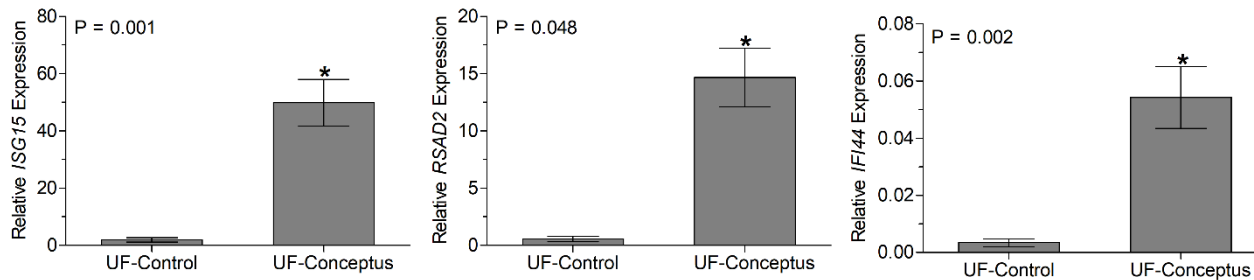
**(A) PBMC**



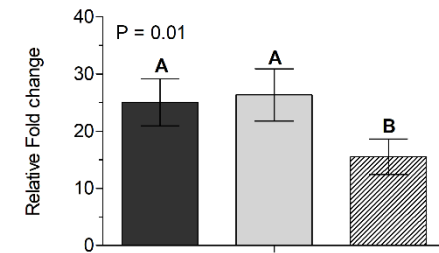
**(C) PBMC**



**(B) PMN**



**(D) PMN**

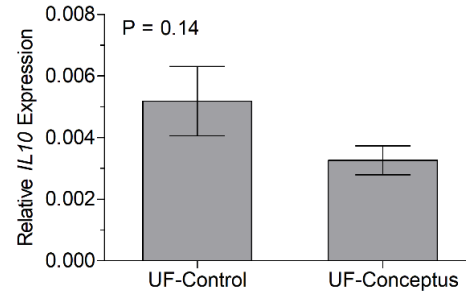
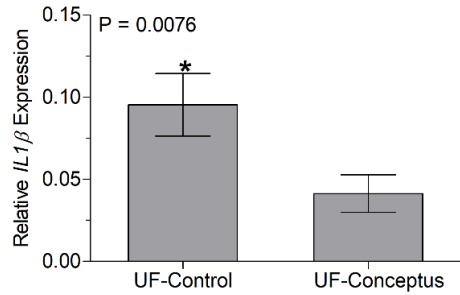


1678

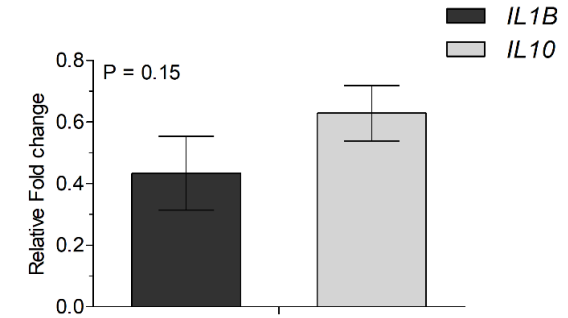
1679 **Figure 4.** Mean  $\pm$  SEM for relative expression and fold change of *ISG15*, *RSAD2* and *IFI44* genes by qPCR in (Panel A, C) PBMC (N=10) cultured for 12 hours  
1680 and (Panel B, D) PMN (N=8) cultured for 3 hours in UF from day-18 of pregnant cows (UF-Conceptus) or UF from non-pregnant cows (UF-Control). <sup>\*AB</sup> An  
1681 asterisk or different letters above the bar indicates a significant difference ( $P \leq 0.05$ ) between the transcripts.

1682

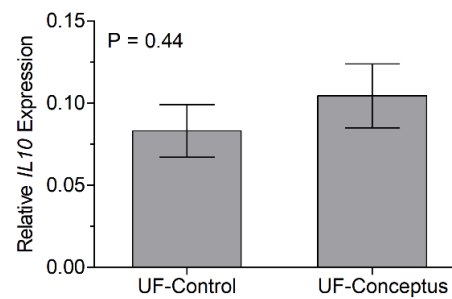
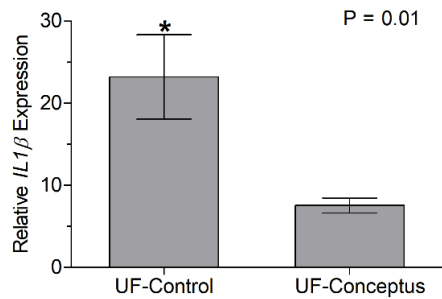
**(A) PBMC**



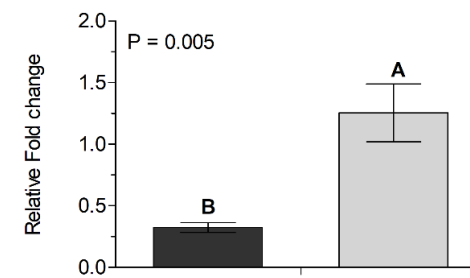
**(C) PBMC**



**(B) PMN**



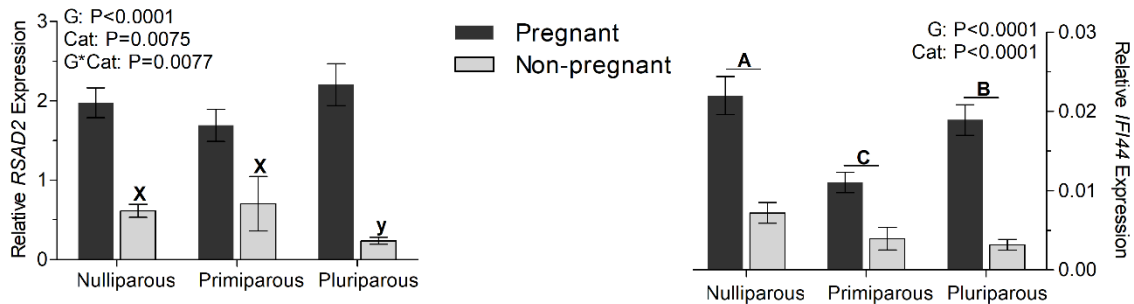
**(D) PMN**



1683

1684 **Figure 5.** Mean  $\pm$  SEM for relative expression and fold change of pro-inflammatory (*IL1β*) and anti-inflammatory (*IL10*) cytokine genes by qPCR in (Panel A,  
1685 C) PBMC (N=10) cultured for 12 hours and (Panel B, D) PMN (N=8) cultured for 3 hours in UF from Day 18 pregnant cows (UF-Conceptus) or UF from non-  
1686 pregnant cows (UF-Control). <sup>\*AB</sup> An asterisk or different letters above the bar indicates a significant difference ( $P \leq 0.05$ ) between the transcripts.

1687

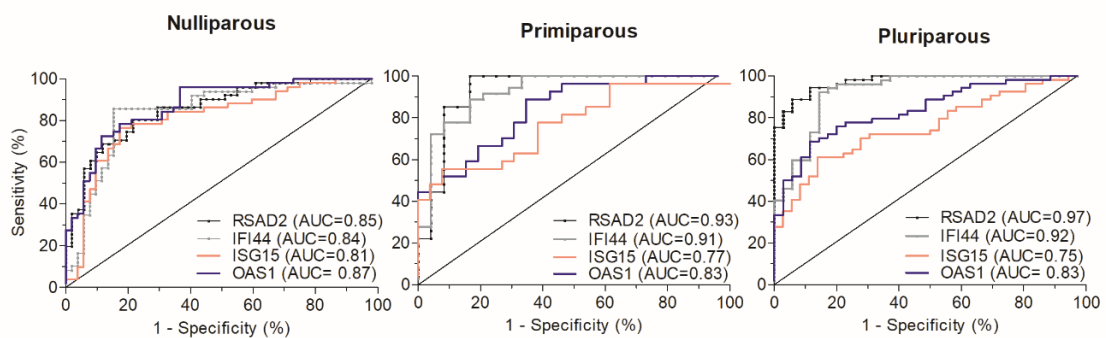


1688

1689 **Figure 6.** Relative expression of *RSAD2* and *IFI44* by qPCR in PMN from pregnant and non-  
1690 pregnant nulliparous (N=103), primiparous (N=53) and pluriparous (N=91) bovine females 20  
1691 days post-timed-AI. The main effects of group (G), category (Cat), and interaction group\*cate-  
1692 gory (G\*Cat) that were significant are shown. <sup>XY</sup> Bars with a different letter indicate a significant  
1693 difference ( $P \leq 0.05$ ) among the parity order in non-pregnant animals. <sup>ABC</sup> Bars with a different  
1694 letter indicate a significant difference ( $P \leq 0.05$ ) among the parity order, regardless of the preg-  
1695 nancy status.

1696

1697



1698

1699 **Figure 7.** ROC (Receiver Operating Characteristic) curves of the classic (*ISG15* and *OAS1*) and  
1700 non-classic (*RSAD2* and *IFI44*) ISGs on D20 post-timed-AI in nulliparous (N=103), primiparous  
1701 (N=53) and pluriparous (N=91) bovine females. The horizontal and vertical axes represent false  
1702 positive rate (1 - specificity) and sensitivity, respectively. Non-classic (*RSAD2* and *IFI44*) ISGs  
1703 provided the most adequate prediction of pregnancy when compared to classic ISGs (*ISG15* and  
1704 *OAS1*) in primiparous and pluriparous bovine females.

### 3.8 TABLES

**Table 1.** Target name, gene number, forward (F) and reverse (R) primer sequence of the genes tested by the qPCR technique

<b>Target Name</b>	<b>Gene Number</b>	<b>Forward primer sequence</b>	<b>Reverse primer sequence</b>	<b>Reference</b>
<i>OAS1</i>	NM_001040606.1	TAGCCTGGAACATCAGGTC	TTTGGTCTGGCTGGATTACC	Shirasuna, et al. [23]
<i>ISG15</i>	NM_174366	GGTATCCGAGCTGAAGCAGTT	ACCTCCCTGCTGTCAAGGT	Oliveira, et al. [24]
<i>RSAD2</i>	NM_001045941.1	TGGTTCCAGAAGTACGGTGAA	ACCACGGCCAATAAGGACAT	Rocha, et al. [19]
<i>IFI44</i>	XM_002686295.6	TCTGCCCATGCTGAAGGAC	CCACATGGACCACATCAGACT	Rocha, et al. [19]
<i>GAPDH</i>	NM_001034034.2	GCCATCAATGACCCCTTCAT	TGCCGTGGGTGGAATCA	Araujo, et al. [25]
<i>ACTB</i>	NM_173979.3	GGATGAGGCTCAGAGCAAGAGA	TCGTCCCAGTTGGTGACGAT	Araujo, et al. [25]
<i>PPIA</i>	BF230516.1	GCCATGGAGCGCTTTGG	CCACAGTCAGCAATGGTGATCT	Pugliesi, et al. [12]

**Table 2.** Pearson's correlation coefficient (r) between the abundance of transcripts in PBMC and PMN culture with recombinant ovine interferon- $\tau$  (roIFNT) or uterine flush (UF).

<i>Endpoint</i>	Between ISGs and Cytokines				<i>Endpoint</i>	ISGs vs Cytokines			
PBMC	IFNT culture		UF culture		PBMC	IFNT culture		UF culture	
	<b>r</b>	<b>P</b>	<b>r</b>	<b>P</b>		<b>r</b>	<b>P</b>	<b>r</b>	<b>P</b>
<i>ISG15 vs RSAD2</i>	0.97	< <b>0.0001</b>	0.81	< <b>0.0001</b>	<i>ISG15 vs IL1<math>\beta</math></i>	-0.26	NS	-0.32	NS
<i>ISG15 vs IFI44</i>	0.94	< <b>0.0001</b>	0.87	< <b>0.0001</b>	<i>ISG15 vs IL10</i>	0.52	<b>0.03</b>	-0.37	NS
<i>RSAD2 vs IFI44</i>	0.96	< <b>0.0001</b>	0.94	< <b>0.0001</b>	<i>RSAD2 vs IL1<math>\beta</math></i>	-0.26	NS	-0.33	NS
<i>IL1<math>\beta</math> vs IL10</i>	-0.04	NS	0.15	NS	<i>RSAD2 vs IL10</i>	0.46	NS	-0.32	NS
					<i>IFI44 vs IL1<math>\beta</math></i>	-0.28	NS	-0.38	NS
					<i>IFI44 vs IL10</i>	0.35	NS	-0.46	<b>0.05</b>
PMN	IFNT culture		UF culture		PMN	IFNT culture		UF culture	
	<b>r</b>	<b>P</b>	<b>r</b>	<b>P</b>		<b>r</b>	<b>P</b>	<b>r</b>	<b>P</b>
<i>ISG15 vs RSAD2</i>	0.96	< <b>0.0001</b>	0.90	< <b>0.0001</b>	<i>ISG15 vs IL1<math>\beta</math></i>	-0.18	NS	-0.50	NS
<i>ISG15 vs IFI44</i>	0.90	< <b>0.0001</b>	0.84	<b>0.001</b>	<i>ISG15 vs IL10</i>	-0.15	NS	0.16	NS
<i>RSAD2 vs IFI44</i>	0.95	< <b>0.0001</b>	0.77	<b>0.006</b>	<i>RSAD2 vs IL1<math>\beta</math></i>	-0.09	NS	-0.51	NS
<i>IL1<math>\beta</math> vs IL10</i>	0.64	<b>0.01</b>	-0.21	NS	<i>RSAD2 vs IL10</i>	-0.07	NS	0.19	NS
					<i>IFI44 vs IL1<math>\beta</math></i>	-0.05	NS	-0.50	NS
					<i>IFI44 vs IL10</i>	-0.08	NS	0.0009	NS

Means indicate differences ( $P \leq 0.05$ ) between treatments.

NS: non-significant.

**Table 3.** Pearson’s correlation coefficient (r) between the abundance of transcripts in PMN on day 20 post-TAI in nulliparous, primiparous, and pluriparous bovine females.

<i>Endpoint</i>	Between ISGs					
	Nulliparous		Primiparous		Pluriparous	
	r	P	r	P	r	P
<i>ISG15 vs OAS1</i>	0.81	< <b>0.0001</b>	0.88	< <b>0.0001</b>	0.88	< <b>0.0001</b>
<i>ISG15 vs RSAD2</i>	0.66	< <b>0.0001</b>	0.20	NS	0.20	NS
<i>ISG15 vs IFI44</i>	0.56	< <b>0.0001</b>	0.44	<b>0.001</b>	0.45	< <b>0.0001</b>
<i>OAS1 vs RSAD2</i>	0.70	< <b>0.0001</b>	0.23	NS	0.16	NS
<i>OAS1 vs IFI44</i>	0.53	< <b>0.0001</b>	0.48	<b>0.0003</b>	0.38	<b>0.0003</b>
<i>RSAD2 vs IFI44</i>	0.73	< <b>0.0001</b>	0.79	< <b>0.0001</b>	0.73	< <b>0.0001</b>

NS: non-significant (P > 0.1).

**Table 4.** Number of True-Positive (TP), True-Negative (TN), False-Positive (FP), False Negative (FN), Sensitivity (SENS), Specificity (SPEC), Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy (ACCU) for determining pregnancy status on D20 post-TAI by *RSAD2* and *IFI44* in nulliparous, primiparous and pluriparous bovine females.

<i>Endpoint</i>	Nulliparous		Primiparous		Pluriparous	
	<i>RSAD2</i>	<i>IFI44</i>	<i>RSAD2</i>	<i>IFI44</i>	<i>RSAD2</i>	<i>IFI44</i>
<i>n</i>	100	100	50	50	83	83
TP ( <i>n</i> )	39	42	27	23	45	46
TN ( <i>n</i> )	40	44	19	19	32	30
FP ( <i>n</i> )	11	7	4	4	1	3
FN ( <i>n</i> )	10	7	0	4	5	4
SENS (%)	79.6	85.7	100.0	85.2	90.0	92.0
SPEC (%)	78.4	86.3	82.6	82.6	97.0	90.9
PPV (%)	78.0	85.7	87.1	85.2	97.8	93.9
NPV (%)	80.0	86.3	100.0	82.6	86.5	88.2
ACCU (%)	79.0	86.0	92.0	84.0	92.8	91.6

<sup>a</sup> Sensitivity (probability that a test result will be positive when the cow is pregnant) = TP/(TP + FN).

<sup>b</sup> Specificity (probability that a test result will be negative when the cow is not pregnant) = TN/(FP + TN).

<sup>c</sup> PPV (probability that the cow is pregnant when the test is positive) = TP/(TP + FP).

<sup>d</sup> NPV (probability that the cow is not pregnant when the test is negative) = TN/(FN + TN).

<sup>e</sup> Accuracy = (TP + TN)/n.

**Table 5.** Number of True-Positive (TP), True-Negative (TN), False-Positive (FP), False-Negative (FN), Sensitivity (SENS), Specificity (SPEC), Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy (ACCU) for determining pregnancy status on D20 post-TAI by *RSAD2*, *IFI44*, *ISG15*, and *OAS1* in bovine females with a functional CL.

<i>Endpoint</i>	<b>Nulliparous</b>				<b>Primiparous</b>				<b>Pluriparous</b>			
	<i>RSAD2</i>	<i>IFI44</i>	<i>RSAD2/IFI44/IFI44</i>	<i>RSAD2/IFI44/ISG15/OAS1</i>	<i>RSAD2</i>	<i>IFI44</i>	<i>RSAD2/IFI44/IFI44</i>	<i>RSAD2/IFI44/ISG15/OAS1</i>	<i>RSAD2</i>	<i>IFI44</i>	<i>RSAD2/IFI44/IFI44</i>	<i>RSAD2/IFI44/ISG15/OAS1</i>
<i>n</i>	100	100	100	100	50	50	50	50	83	83	83	83
VP ( <i>n</i> )	39	42	44	48	27	23	27	27	45	46	48	48
VN ( <i>n</i> )	47	47	46	43	22	22	22	21	32	30	30	28
FP ( <i>n</i> )	4	4	5	8	1	1	1	2	1	3	3	5
FN ( <i>n</i> )	10	7	5	1	0	4	0	0	5	4	2	1
SENS (%)	79.6	85.7	89.8	98.0	100.0	85.2	100.0	100.0	90.0	92.0	96.0	98.0
SPEC (%)	92.2	92.2	90.2	84.3	95.6	95.6	95.7	91.3	97.0	90.9	90.9	84.8
PPV (%)	90.7	91.3	89.8	85.7	96.4	95.8	96.4	93.1	97.8	93.9	94.1	90.7
NPV (%)	82.5	87.0	90.2	97.7	100.0	84.6	100.0	100.0	86.5	88.2	93.7	96.5
ACCU (%)	86.0	89.0	90.0	91.0	98.0	90.0	98.0	96.0	92.8	91.6	94.0	92.8

<sup>a</sup>Evaluation of *RSAD2*, *IFI44*, *ISG15*, and *OAS1* in females with a functional CL was performed by applying the predefined cutoffs only in females in which CL blood perfusion was > 25% on D20 post-TAI.

<sup>b</sup>The combined use of both genes (*RSAD2*, *IFI44*, *ISG15*, and *OAS1*) was performed by considering the female as pregnant when the expression levels of at least one gene were greater than the predefined cutoffs.

**Table 6.** Number of True-Positive (TP), True-Negative (TN), False-Positive (FP), False Negative (FN), Sensitivity (SENS), Specificity (SPEC), Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy (ACCU) for determining pregnancy status on D20 post-TAI by *RSAD2*, *IFI44*, *ISG15* and *OAS1* in bovine females with a functional CL.

<i>Genes</i>	<i>ISG15</i>			<i>OAS1</i>		<i>RSAD2</i>	<i>RSAD2/IFI44/</i>
	<i>OAS1</i>	<i>RSAD2</i>	<i>IFI44</i>	<i>RSAD2</i>	<i>IFI44</i>	<i>IFI44</i>	<i>ISG15/OAS1</i>
<i>n</i>	233	233	233	233	233	233	233
TP ( <i>n</i> )	108	115	118	122	123	120	124
TN ( <i>n</i> )	92	95	95	95	95	98	92
FP ( <i>n</i> )	15	12	12	12	12	9	15
FN ( <i>n</i> )	18	11	8	4	3	6	2
SENS (%)	85.7	91.3	93.6	96.8	97.6	95.2	98.4
SPEC (%)	86.0	88.8	88.8	88.8	88.8	91.6	86.0
PPV (%)	87.8	90.5	90.8	91.0	91.1	93.0	89.2
NPV (%)	83.6	89.6	92.2	96.0	96.9	93.2	97.9
ACCU (%)	85.8	90.1	91.4	93.1	93.6	93.6	92.7

<sup>a</sup> Evaluation of *ISG15*, *OAS1*, *RSAD2*, and *IFI44* in females with a functional CL was performed by applying the predefined cutoffs only in females in which CL blood perfusion was >25% on D20 post-TAI.

<sup>b</sup> The combined use of both genes (*RSAD2*, *IFI44*, *ISG15*, and *OAS1*) was performed by considering the female as pregnant when the expression levels of at least one gene were greater than the predefined cutoffs.

### 3.9 SUPPLEMENTARY TABLES

**Table 1.** Pre- and post-culture cell viability of PBMC and PMN treated (100 ng/mL roIFNT) or untreated (Control) with recombinant ovine interferon- $\tau$  (roIFNT).

<b>PBMC Culture</b>			
<b>Sample</b>	<b>Treatment Group</b>	<b>Viability Pre-Culture (%)</b>	<b>Viability Pos-Culture (%)</b>
2956	Control	91	90
	IFNT		94
4162	Control	94	90
	IFNT		92
4853	Control	98	92
	IFNT		87
5823	Control	96	89
	IFNT		86
6016	Control	97	87
	IFNT		93
6058	Control	93	97
	IFNT		90
6134	Control	97	87
	IFNT		88
6409	Control	98	87
	IFNT		84
6652	Control	97	86
	IFNT		91
<b>PMN Culture</b>			
1417	Control	99	90
	IFNT		74
1784	Control	89	90
	IFNT		88
2956	Control	95	97
	IFNT		88
4294	Control	92	76
	IFNT		74
4340	Control	97	93
	IFNT		92
4135	Control	97	82
	IFNT		95

6253	Control	96	85
	IFNT		93
1784	Control	96	92
	IFNT		94
3500	Control	98	93
	IFNT		94
6504	Control	92	81
	IFNT		86
6221	Control	95	88
	IFNT		92
6016	Control	96	84
	IFNT		97

**Table 2.** Pre- and post-culture cell viability of PBMC and PMN cultured in UF from Day 18 of pregnant cows (UF-Conceptus) or UF from non-pregnant cows (UF-Control).

<b>PBMC Culture</b>			
<b>Sample</b>	<b>Treatment Group</b>	<b>Viability Pre-Culture (%)</b>	<b>Viability Pos-Culture (%)</b>
1524	UF-Control	94	85
	UF-Conceptus	97	88
1833	UF-Control	97	80
	UF-Conceptus	96	83
4162	UF-Control	97	81
	UF-Conceptus	97	75
5480	UF-Control	96	68
	UF-Conceptus	95	84
5823	UF-Control	98	77
	UF-Conceptus	97	83
5858	UF-Control	96	91
	UF-Conceptus	99	96
6058	UF-Control	96	77
	UF-Conceptus	97	91
6134	UF-Control	94	84
	UF-Conceptus	95	81
6253	UF-Control	97	88
	UF-Conceptus	98	92
6504	UF-Control	98	91
	UF-Conceptus	97	83
<b>PMN Culture</b>			

4294	UF-Control	96	96
	UF-Conceptus	100	93
4340	UF-Control	92	93
	UF-Conceptus	95	97
6409	UF-Control	99	96
	UF-Conceptus	98	97
1784	UF-Control	100	94
	UF-Conceptus	95	94
6003	UF-Control	100	98
	UF-Conceptus	97	98
6058	UF-Control	94	100
	UF-Conceptus	100	96
1784	UF-Control	95	100
	UF-Conceptus	96	95
6003	UF-Control	97	100
	UF-Conceptus	99	93