REPRODUCTIVE EFFICIENCY OF WAGYU DONOR COWS SUPPLEMENTED WITH A MIXTURE OF LIVE YEAST AND ORGANIC MICROMINERALS

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ABSTRACT
Strengthening the beef production chain depends on increasing the number and quality of calves born, which in turn relies heavily on adequate maternal nutrition. This experiment was carried out to evaluate efficiency of ovum pick-up and in vitro embryo production in Wagyu breed oocyte donor cows with and without a supplemented mixture of live yeasts and organic microminerals. Wagyu breed oocyte donor cows (n=14; BW=370kg) were divided into two experimental groups: Control Group - animals that received a conventional diet; and Test Group - animals that received a conventional diet supplemented with 2 g of Advantage Pasto®/100 kg of body weight/day. All animals were submitted to four synchronization protocols, followed by follicular aspiration, with an average interval of 30 days. The results showed a greater ($P<0.05$) number of total and viable oocytes for the donors supplemented with the mixture of live yeasts and organic microminerals after 90 days of supplementation.

KEYWORDS: natural additive; reproductive biotechnology; cattle nutrition.

1. INTRODUCTION

In global beef production Brazil stands out as having the largest commercial herd of beef cattle and being the top exporter of fresh beef (ABIEC, 2023). However, reproductive rates in Brazilian beef cattle are low compared to other countries (DUBON et al., 2021) indicating an area in need of attention to continue to improve the beef supply chain.

Adequate nutritional management of cows can contribute to increasing herd reproductive efficiency and the number and quality of calves born. (MENDONÇA JÚNIOR al., 2011; COUTO et al., 2017) Studies show that nutritional changes can interfere with follicular growth patterns, endocrine parameters, circulating concentrations of steroids and the secretory activity of the bovine uterus (ROBINSON et al., 2006; LEROY et al., 2008). Supplementation with organic microminerals and/or microbial yeasts can also influence follicular dynamics, *in vitro* embryo production (WINGERT et al., 2013), and the number of viable oocytes collected (TURNBULL, 1993). Thus, maternal nutrition plays a key role in the success of techniques such as *in vitro* embryo production (IVP), a biotechnique of prominence that increases the rates of calves born from genetically superior animals and improves herd productivity (VIANA; CAMARGO, 2007; DOMINGUES et al., 2014).

However, maternal nutrition and fertility have a complex relationship. Based on previous studies, the effects of micronutrients and feed additives on reproduction biotechnique outcomes are inconsistent, variable (BOLAND et al., 2001), little understood (DAWSON, 2006) and scarce, especially in relation to in beef cows. Thus, this experiment was conducted with the aim of evaluating the efficiency of *ovum pick-up* and *in vitro* embryo production Wagyu breed oocyte donor cows fed a mixture of live yeasts and organic microminerals.

2. MATERIALS AND METHODS

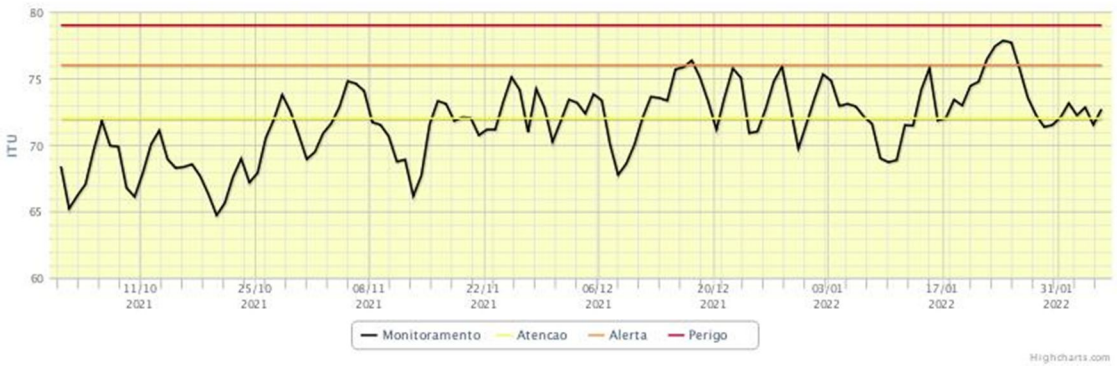
The experiment was carried out from September 2021 to January 2022 at the Farm School of Cesumar University/UNICESUMAR, Maringá, northwest region of Paraná, Brazil (23°25'S, 51°57'W and altitude of 550 meters). The region has a temperate subtropical climate, type Cfa, according to the classification of Köppen and Geiger. Data referring to the Temperature and Humidity Index (THI) of the experimental period, an indicator of the thermal comfort condition for cattle, can be seen in Figure 1.

The methods employed in this research were approved by the Ethics Committee on the Use of Animals of Cesumar University/UNICESUMAR.

Fourteen Wagyu oocyte donor cows aged 2 to 4 years and mean body weight of 370 kg were used. The animals were distributed in a completely randomized design, in two experimental groups, with seven repetitions each. Treatmetn groups were as follows:

1) Control group – animals that received the conventional diet and;
2) Test group - animals that received the conventional diet supplemented with Advantage Pasto® (Alltech of Brazil Agroindustrial, Maringa, Parana, Brazil).

Figure 1. Temperature and Humidity Index (THI) values during the experimental period – October/2021 to January/2022.



Source: SISDAGRO (2023)

Advantage Pasto® is a nutritional additive with the following guarantee levels (minimum): 41.25 g/kg of mannanoligosaccharides, 22.60 g/kg of beta-glucans, 1.5 x 1011 CFU/kg from *Saccharomyces cerevisae* strain 1026, 30 g/kg of zinc in the form of zinc proteinate, 15 g/kg of manganese in the form of manganese proteinate, 8.75 g/kg of copper in the form of copper proteinate, 6.25 g/kg of iron in the form of iron proteinate, 0.625 g/kg of iodine in the form of potassium iodate, 0.25 g/kg of chromium in the form of chromium yeast, 0.19 g/kg of cobalt in the form of cobalt proteinate and 0.125 g/kg of selenium in the form of selenium-enriched yeast.

The animals were kept on pasture, in paddocks with *Cynodon dactylon* (L.) Pers cv. Coast-cross, supplemented in the trough, twice a day, at 8 am and 4 pm, with 10 kg of corn silage (dry matter) and 1.0 kg of concentrate, composed of 61.4% ground corn,

27.4% soybean meal, 5% urea and 6.2% calcitic limestone, containing 16% crude protein and 70% total digestible nutrients (TDN).

Advantage Pasto® was added after supplying the diet to the animals for the test group at a dose of 2 g/100 kg of body weight/day following the manufacturer's instructions, which resulted in an approximate daily supply of 7.4 g/cow /day.

The 1st synchronization protocol took place on a random day of the estrous cycle, and shortly after the 1st follicular aspiration session, the supply of Advantage Pasto® was started for the animals in the test group; thus, the 1st session was considered a control session for both groups. After this session, all animals were submitted to three more consecutive synchronization protocols, followed by follicular aspiration (*ovum pick-up* - OPU), with an average interval of 30 days.

The synchronization protocol was initiated with intramuscular application of 2 mg of estradiol benzoate and the introduction of an intravaginal progesterone device (CIDR®; Zoetis, São Paulo, SP). After nine days, the device was removed and 150 µg of sodium D-cloprostenol, 300 IU of equine chorionic gonadotropin and 1.0 mg of estradiol cypionate were administered intramuscularly. The day of ovulation was considered 48 hours after removal of the progesterone device. The donors underwent follicular aspiration eight days after the day of ovulation.

Follicular aspiration and oocyte retrieval were performed using an ALOKA SSD-500 ultrasound with a 5 MHz microconvex transducer (UST 974-5), adjusted to a specific follicular aspiration guide for the bovine reproductive system. A 20G needle was used, connected to a 50 mL Falcon tube through an aspiration system (Cook VBOA 18L®). Vacuum pressure was obtained by a Cook V-MAR 5000 pump, adjusted between 38 and 45 mmHg, allowing a flow of 12 mL of medium/minute.

The oocytes were aspirated into a solution containing 2.0% Fetal Bovine Serum (FBS; Nutricell®), 25 IU/mL of sodium heparin and 98.0% PBS (Phosphate buffered saline; Nutricell®). The animals were submitted to low epidural anesthesia with 5 mL of 2% lidocaine (Pearson®) and, soon after, the vulva was washed and wiped with a paper towel. Next, the 5 mHz microconvex transducer (UST 974-5) was inserted up to the vaginal cul-de-sac in order to obtain the best view of the ovaries on the ultrasound screen in which the manipulation was performed via the rectum.

Then, the follicles were positioned on the puncture line indicated on the ultrasound screen and aspirated through the needle and vacuum pump. At the end of the aspiration, the vacuum system was cleaned with the medium for receiving the oocytes and the needles were discarded.

For washing and selection of oocytes, the aspirated material was transferred to the embryo collection filter and washed with the same medium used for aspiration. The sediments remaining on the filter were transferred to a Petri dish and the oocytes were identified and counted. Classification of the oocytes was carried out as follows: total, viable and non-viable. Those being considered viable that presented the presence of

homogeneous cumulus and ooplasma, and non-viable those that were naked or pyknotic, heterogeneous and with apoptotic vesicles (DE LOOS et al., 1989).

Embryo production was carried out at the Animal Reproduction Biotechnology Laboratory (BIOTEC/UNICESUMAR). The selected cumulus-oocyte complexes (COCs) were washed in TCM199 supplemented with Hepes and placed on plates with TCM199 maturation medium with Earles salts (Gibco®), glutamine (Sigma® VPN: G8540) and $NaHCO_3$ (Mallinckrodt®), supplemented with 10% fetal bovine serum (FBS; Cultilab®), 22 µg/mL pyruvate (Biochemical® VPN: 44094), 50 µg/mL gentamicin (Sigma® VPN: G1272), 0.5µg FSH/mL (Bioniche®), 50 µg of LH/mL (Bioniche®) and 1 µg of estradiol/mL (Sigma® VPN: E2758) and kept in an oven, at 38.5°C of average temperature, 5% of CO_2 in air and with maximum humidity for 22 to 24 hours.

The oocytes were placed in microdrops of 75 µL of maturation medium covered with mineral oil. Then, the matured oocytes were submitted to *in vitro* fertilization (IVF) using TALP medium in which 10 µg/mL of heparin (Sigma® VPN: H3149), 22µL/mL of pyruvate (Biochemical® VPN: 44094), 50 µg /mL of gentamicin (Sigma® VPN: G1272), bovine serum albumin (BSA, without fatty acids; Sigma® VPN: A3311), PHE solution with 2µM penicillin (Sigma® VPN: P4875), 1 µM hypotaurine (Sigma® VPN: H1384) and 0.25 µM epinephrine (Sigma® VPN: E4250) were added.

For fertilization, semen from same a Wagyu bull was used, which was thawed in a water bath at 36°C. For selection of motile spermatozoa and removal of extenders and seminal plasma, centrifugation was performed in Percoll gradient (90% and 45%) for 5 minutes. The insemination dose was $1x10^6$ sperm/mL and the oocytes were transferred to the microdroplets (20 oocytes/drop), where they remained for 22 to 24 h, at 38.5°C, in an atmosphere with 5% CO_2 in air.

After fertilization, the zygotes were cultured *in vitro* (IVC), in SOF medium (*Synthetic Oviduct Fluid*) supplemented with 5% FBS (Cultilab®), in an incubator with a closed atmosphere in a special mixture with $CO_2/N_2/O_2$.

After 48 hours, the cleavage rate (n^o cleaved/n^o of oocytes) was evaluated and the culture medium was renewed. At that time, embryonic development with two, four and eight cells was observed. Then, the embryos were placed in a closed atmosphere for continued development.

On the 7^{th} day of the IVC, the number of grade I blastocysts was evaluated, considering structures of good or excellent quality, that is, with few damaged blastomeres, with a maximum of 15% of cells extruded from the cell mass of the embryo, according to the criteria of evaluation of the Manual of the International Society of Embryo Transfer (STRINGFELLOW; SEIDEL, 1998). On the 10^{th} day of culture, the total number of embryos (hatched blastocysts) was evaluated.

The variables of OPU efficiency, viability rate (percentage of viable oocytes in relation to total oocytes) and blastocyst rate (percentage of embryos in relation to total viable oocytes) were evaluated in the same animals throughout the experiment. Therefore, all data were considered as repeated measures over time and were analyzed as mixed

models using the MIXED procedure of SAS (version 9.4). Collections every 30 days were considered as a fixed effect, while animals were considered as a random effect. The first-order autoregressive heterogeneous matrix (ARH (1)) was used for analysis of variance and covariance matrix of random effects for all variables. For comparisons, the 0.05 level was established as a critical level of probability for the occurrence of type I error.

3. RESULTS & DISCUSSION

The results presented in Table 1 show differences in the number of nonviable oocytes after 30 days of Advantage Pasto® supply and the number of total and viable oocytes after 90 days of Advantage Pasto® supply ($p<0.05$). The other measured variables of OPU and PIVE efficiency were not different between the two experimental groups ($P>0.05$; Table 1).

The observed greater number of total and viable oocytes at 90 days demonstrates the expected effect of supplementation with the commercial additive, since the effect of any nutrient on reproduction is only evidenced after 90 days of consumption.

In cattle, primordial follicle growth, once initiated, persists until ovulation or atresia occurs (MARQUES et al., 2012). The mechanisms that control the initiation and number of primordial follicles recruited for growth are not well understood, but there is evidence that the period required for the primordial follicle to reach the preovulatory stage is approximately 90 days (CAMPBELL; SCARAMUZZI; WEBB, 1995).

Table 1. Mean efficiency of ovum pick-up and in vitro embryo production of Wagyu cows, oocyte donors, fed a diet with and without Advantage Pasto® (mixture of live yeasts and organic microminerals) according to the collection sessions.

Variable	Control group	Test group	MSE	P value
1º session – 0 day				
Total oocytes (n)	14.57	17.29	3.54	0.5974
Viable oocytes (n)	12.29	14.86	3.50	0.6127
Non-viable oocytes (n)	2.29	2.29	0.28	1.0000
Viability rate (%)	78.44	82.19	4.99	0.6048
Embryos (n)	5.57	5.57	1.87	1.0000
Blastocyst rate (%)	50.13	48.67	5.58	0.8509
2º session – 30 days				
Total oocytes (n)	13.14	18.57	2.98	0.2228
Viable oocytes (n)	11.57	13.86	2.60	0.5461
Non-viable oocytes (n)	1.57	4.71	0.75	0.0118
Viability rate (%)	83.45	73.87	4.18	0.1300
Embryos (n)	4.50	4.00	1.39	0.8040
Blastocyst rate (%)	34.82	25.69	5.89	0.2992

3º session – 60 days				
Total oocytes (n)	12.14	18.57	2.35	0.0772
Viable oocytes (n)	9.00	12.29	1.95	0.2565
Non-viable oocytes (n)	3.14	6.29	1.54	0.1737
Viability rate (%)	77.71	67.69	7.65	0.3724
Embryos (n)	3.29	4.00	0.96	0.6084
Blastocyst rate (%)	40.07	34.48	6.89	0.5765
4º session – 90 days				
Total oocytes (n)	9.80	19.20	2.45	0.0264
Viable oocytes (n)	8.80	16.00	2.09	0.0409
Non-viable oocytes (n)	2.50	3.20	1.06	0.6018
Viability rate (%)	92.31	83.07	3.89	0.1321
Embryos (n)	3.00	8.25	2.62	0.2063
Blastocyst rate (%)	35.97	47.92	9.12	0.3904

Control group – animals that received the conventional diet; Test group - animals that received a conventional diet plus 11 grams/day of additive Advantage Pasto®. MSE: mean standard error.

During initial development primordial follicles grow under the influence of growth factors, secreted by the cells that surround the oocyte, and from the formation of the follicular antrum, they become dependent on gonadotropins (WEBB et al., 2004; MELLO et al., 2014; SENEDA et al., 2021). During this period, in addition to local growth factors and gonadotropins, follicles can be influenced by external factors, such as nutrition (LEROY et al., 2008).

Knowledge of the effects of nutrition on the initial development of follicles is still scarce, mainly due to the lack of in-depth information on the factors that trigger the growth of primordial follicles (MARQUES et al., 2012). However, there are studies that evaluated the effects of nutrition on the final development of the follicle, from the emergence of the follicular wave to ovulation (STAGG et al., 1995; SCARAMUZZI et al., 2006). Final follicular growth can be influenced by both a shortage or excess of nutrients (MARQUES et al., 2012).

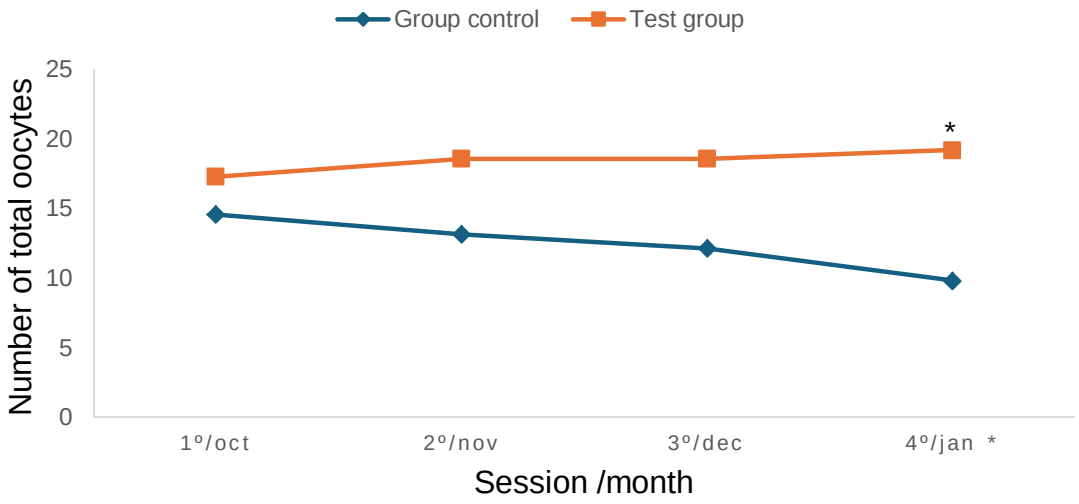
The nutritional and metabolic status of the cow can alter follicular growth patterns, endocrine parameters, circulating concentrations of steroids and secretory activity of the bovine uterus (ROBINSON et al., 2006; LEROY et al., 2008). In a study conducted by Wingert et al. (2013), the authors evaluated the influence of supplementation with microminerals (Zn, Cu and Se) on follicular dynamics and *in vitro* embryo production in Nelore cows and observed an increase in the diameter of ovulatory follicles and better *in vitro* maturation and embryonic rate of cleavage. Investigating the effects of replacing the inorganic mineral supplement with microminerals in the form of proteinate and microbials in beef cows, TURNBULL (1993) observed a marked improvement in the number of viable oocytes collected. Thus, the results of this trial can likely be attributed mainly to the presence of organic microminerals in the additive.

Another interesting result observed in this study was the better resistance of the animals in the face of challenges, mainly environmental ones, imposed in the final period of the experiment. According to the Agricultural Decision Support System (SISDAGRO), developed by the National Institute of Meteorology (INMET) which evaluates Bovine Thermal Comfort, the animal needs satisfactory environmental conditions for its physiological processes. Thus, the Temperature and Humidity Index (THI) is a good indicator of the thermal comfort condition (SISDAGRO, 2023). In fact, the most common and appropriate measure to assess heat stress is the THI, which considers the combined effects of ambient temperature and relative humidity (BOHMANOVA; MISZTAL; COLE, 2007), being a useful and simple way to assess risk of thermal stress (AKYUZ; BOYACI; CAYLI, 2010).

The animals *Bos taurus* undergo thermal stress at THI 70, with thermoregulatory mechanisms ceasing to be effective above THI 80 (LEMERLE; GODDARD, 1986). Generally mild heat stress in cattle starts with a THI of 72, increasing to moderate levels of 79 and severe levels of 89 (AKYUZ; BOYACI; CAYLI, 2010). THI values greater than 72 impose discomfort to the animal and affect its performance. The discomfort is greater with the increase in the index, with 72 being a value of attention, 76 of alert and 79 of danger, being an effective risk to the health of the herd (SISDAGRO, 2023).

Thus, when we analyze the number of total oocytes produced as a function of the months of the experiment (Figure 2), it is verified that the donors supplemented with the commercial additive expressed, through this variable, better resistance to the thermal discomfort imposed, especially in the months from December to January. According to the data published by SISDAGRO, collected by the meteorological station of Maringá, Paraná, the months of December 2021 and January 2022 presented THI above 72, with some points above 75, considered alert points (SISDAGRO, 2023).

Figure 1. Mean number of oocytes collected (n), as a function of *ovum pick-up* sessions in Wagyu breed oocyte donor cows, fed a diet with and without a mixture of live yeasts and organic microminerals.



* P<0,005

In addition, it is known that in the search for better meat production and carcass quality, some taurine breeds recently introduced in Brazilian territory such as the Wagyu, the breed of the females in this study, may suffer more intensely from climate variations in tropical and subtropical regions (FLORES et al., 2019). This information further corroborates the beneficial effect promoted by the evaluated supplementation.

Finally, we mentioned that the general average of total oocytes produced by the test group was higher when compared to the control group (17.99 vs 12.13; $p<0.05$). Therefore, we associate this better general average with better feed efficiency promoted by Advantage Pasto®, improvement of biochemical and physiological functions, which resulted, among other actions, in better immunity and greater protection and resistance of the animals against the stress caused by the high THI and, therefore, better reproductive performance (ALLTECH, 2023) compared to control group donors.

4. CONCLUSIONS

The results of this study showed greater total and viable oocyte numbers for donor cows supplemented with Advantage Pasto®, composed of live yeasts and organic microminerals.

The data also revealed that the offer of Advantage Pasto® improved the resistance to thermal discomfort of the donors, since the moments of the 3rd and 4th sessions were

characterized with THI alert points, and even under these circumstances the supplemented donors maintained the number of total and viable oocytes.

ACKNOWLEDGEMENTS

To the Cesumar Institute of Science, Technology and Innovation (ICETI) for the financial support and to Alltech do Brasil Agroindustrial Ltda., for making Advantage Pasto available to conduct the research.

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