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Use of a commercial feed supplement based on diatom earth and yeast products on oxidative status and in vitro immune response in buffaloes during peripartum

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Abstract

The transition period is a critical metabolic phase for dairy ruminants, especially those with high production levels. In spite of this, little is still known about dairy water buffalo. The aim of this study was to evaluate the effect of a commercial feed additive based on diatomaceous earth and hydrolyzed yeasts on health status, milk quality, and immune response of buffalo cows during the transition period. Eighty healthy Water buffaloes (*Bubalus bubalis*) of Italian Mediterranean breed were included in the trial. They were subdivided into two groups: one group received the additive ($n = 40$) while the control group ($n = 40$) received a placebo. The trial lasted 120 d, from 60 d before calving to 60 d in milk. Blood samples were collected from each buffalo at −60 (60 d from the expected calving), −30, 0 (calving), +15, +30, and +60 d (respectively, i.e., 15, 30, and 60 d in milking). The biochemical as well as the oxidative profile, and the antioxidant power and enzymatic activity were evaluated in the samples obtained. Moreover, acute phase proteins, reactive proteins, and interleukin plasma levels were determined. Peripheral blood mononuclear cells (**PBMCs**) and monocytes were isolated and viability, reactive oxygen species (**ROS**), and reactive nitrogen species were measured on PBMC and monocytes. The introduction of additives enhanced the total antioxidant capacity and enzyme activity, while no differences were observed in oxidation products throughout the trial. Additionally, it significantly reduced the synthesis of ROS in polymorphonuclear cells, supporting a potential positive response in animals experiencing inflammation. The impact of oxidation on the products was not evident. Despite higher enzyme levels in plasma, this did not necessarily correspond to significantly increased enzymatic activity but rather indicated a higher potential. From these results, it was evident that the transition period in buffaloes differs notably from what reported in the literature for cows, probably due to the absence of common postpartum production diseases in dairy cows and lower metabolic challenges linked to lower milk production in buffaloes. Few parameters exhibited notable changes during the transition period in buffaloes, notably certain antioxidant enzymes, PBMC viability, PBMC ROS production, and Hp levels.

Lay Summary

The findings of this paper on the use of diatomaceous earth and yeast products during the transition period in buffaloes reveal that their inclusion does not significantly affect milk production, both qualitatively and quantitatively, or the overall health status of the animals. However, intriguingly, results pertaining to oxidative status and peripheral blood cells, stimulated ex vivo, indicate that even in the absence of pronounced stress during the peripartum period, the animals exhibit increased potential antioxidant response. These insights suggest a potential for enhancing physiological responses in transition period buffaloes, opening avenues for further research on the nuanced impacts of these additives and their implications for animal well-being.

Keywords: dairy buffalo, inflammatory markers, oxidative status, diatom earth

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BHB: β-hydroxybutyrate; CAT, catalase; CON, control group; CRP, C-reactive protein; DCFH-DA, 20,70-dichlorofluorescein diacetate; DEC, additive group; DIM, days in milk; DM, dry matter; DNPH, 2,4-dinitrophenyl hydrazine; DSCC, differential somatic cell count; DSCS, differential somatic cell score; FRAP, ferric reducing antioxidant power; GPx, glutathione peroxidase; Hp, haptoglobin; IL, interleukin; MDA, malondialdehyde; NDF, neutral detergent fiber; PBMCs, peripheral blood mononuclear cells; PMN, peripheral mononuclear cells; RNS, reactive nitrogen species; ROS, reactive oxygen species; RP, reactive protein; SCC, somatic cell count; SCS, somatic cell score; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TMR, total mixed ration.

Introduction

The global demand for reducing the use of antibiotics in the livestock industry has sparked increased interest in nutraceutical feed additives as a potential strategy for the prevention of diseases and improving the health and performance of dairy animals (Ballou et al., 2019; Peng et al., 2020; Gunun et al., 2022b). However, there is a need for scientific evidence capable of assessing the effectiveness of each feed additive. Although antibiotics have historically been used for infectious disease treatment (Van et al., 2020), a significant portion of them has been utilized until their ban (January 2006) for nontherapeutic purposes such as growth promotion (Duffield et al., 2012; Chattopadhyay, 2014). Nutraceutical compounds, as they are commonly

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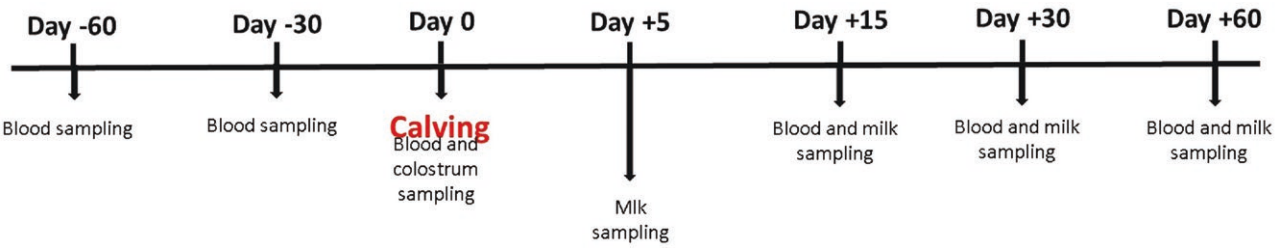

Figure 1. Timing of sampling in buffaloes treated with dietary supplementation of Decosel (DEC) and untreated buffaloes (CON).

referred to, comprise natural compounds and/or microbes that potentially induce advantageous effects related to ruminant health and productivity, including improved feed efficiency, milk production, and disease resistance (Ballou et al., 2019). Given the prevalence of antibiotic resistance and concerns about its future global impact (Smith et al., 2020), along with public apprehensions regarding the potential link between zoonotic multidrug-resistant bacterial strains and antibiotic use in food-producing animals, nutraceutical products have gained importance as alternatives for promoting animal health, rumen fermentation, growth performance and reduce susceptibility to diseases (Lopreiato et al., 2020; Besharati et al., 2022; Gunun et al., 2022a; Maggiolino et al., 2022). Numerous studies have been conducted to explore the potential effects of nutraceutical feed additives like enzymes (Morsy et al., 2016), yeasts (Khattab et al., 2010), and phytogenic additives (Samal et al., 2018; Lakhani et al., 2019; Chanu et al., 2020; Kumar et al., 2022) on growth, production efficiency, rumen fermentation and gas emission, and oxidative and immune status of buffalo calves and dairy buffalo cows.

To the best of our knowledge, no studies have been conducted on the effect of diatomaceous earth (**DE**) in dairy buffaloes. DE is a dust composed of the fossilized bodies of unicellular algae called diatoms, existing naturally as soft chalky rock deposits. It is prepared for commercial use through quarrying, drying, and milling processes, which reduce its moisture content and mean aggregate particle size. DE is a generic term for various silicas and consists in 80% and 95% pure amorphous silicon dioxide along with some minerals including calcium, iron, magnesium, sodium, copper, zinc, selenium, manganese, phosphorus, cobalt, rubidium, etc. Additionally, the use of DE in ruminants, including calves and dairy cows, has primarily been investigated for its anthelmintic and antiparasitic effects (Santos et al., 2021; Zeni et al., 2021). Some studies have reported that dietary inclusion of DE enhances the health status and weight gain in cattle (Milton and Klopfenstein, 2000), as well as in nonruminants animals as broiler (Kayumu, 2008).

To the best of our knowledge, there is a lack of information regarding the antioxidant and anti-inflammatory potential of DE as an additive in buffaloes, especially during the transition period, and its potential effects on the immune system are unknown. The buffalo population plays a crucial role in Southern Italy economy for the production of milk and dairy products such as PDO mozzarella Buffalo cheese. However, the milk quality of this species and the reproductive efficiency are often compromised by harsh environmental conditions due to intensive farming systems, photoperiod, feed quality, and availability (Perera, 2011).

We hypothesized that oral administration of DE along with hydrolyzed yeast and yeast walls during peripartum could improve health status, potentially addressing specific physiological conditions including oxidative stress and immune challenges.

Therefore, the present study aimed to investigate the effects of a commercial feed additive based on DE and hydrolyzed yeasts on health status, milk quality, and immune response of buffalo cows during the transition period. This period was chosen due to its challenging nature, which makes it suitable for detecting potential evidence of the additive's effects.

Materials and Methods

The trial was authorized by the Ethical Committee for Animal Welfare of Animals employed in scientific research of the Department of Veterinary Medicine of the University of Bari (approval no. 07/2020).

Animal management and experimental design

The study was carried out in a commercial farm in southern Italy (Fattoria Apulia, 41.444 N, 15.914 E). Eighty healthy water buffaloes (*Bubalus bubalis*) of Italian Mediterranean breed, both primiparous ($n = 24$) and multiparous ($n = 66$), were included in the trial. They were subdivided into two separated groups, balanced for parity (2 to 6 lactations; 3.69 ± 1.30, mean ± SD) and milk yield of the previous lactation (only for multiparous). A group was the treatment group and received the additive (DEC; $n = 40$; 60 g/head/d of Decosel, Agroteam S.p.A, Italy), as the manufacturer indications for buffalo cows, while the control group (CON; $n = 40$) received a placebo.

The additive contained DE, hydrolyzed yeast (*Saccaromices spp.*), yeast walls, barley meal, and $CaCO_3$ as support. The placebo contained the same amount of barley meal and $CaCO_3$ administered to DEC group. All buffaloes have been subjected to estrus synchronization and artificial insemination within 7 d and all enrolled animals calved in a range of 20 d (from first to last calving, Figure 1). During the whole experimental period, animals were reared in two different outdoor barns, one for dry animals and one for lactating animals, regardless of treatment or parity classification, provided with a shadow area (not less than 6 m^2/head) and fed with same ration administered as total mixed ration (**TMR**) once a day. Every day refusal was measured for each group with the aim of mean dry matter (**DM**) intake calculation. The refusal was always less than 2% of the total administered. Animals were fed with two different diets according to their physiological status, a diet for dry animals and one for lactating ones (Table 1). All the animals were daily monitored by the farm veterinarian during the whole trial and no animal incurred

Table 1. Ingredients and nutrient composition of different experimental diets fed to buffaloes with Decosel (DEC) supplementation and without it (CON) during the trial before calving (dry period) and after calving (lactation period)

Ingredients	Lactation period		Dry period	
	Kg (kg WB[1]/head)	% WB	Kg (kg WB/head)	% WB
Corn silage	19	60.3	6.5	42.8
Oat hay	3.6	11.4	—	—
Barley straw	—	—	5.5	36.2
Soybean meal 48%	2.1	6.7	0.3	2.0
Cornflour	2	6.3	0.9	5.9
Alfalfa hay	1.6	5.1	—	—
Fat	1	3.2	1.8	11.8
Sunflower seeds	0.6	1.9	—	—
Flaked corn	0.6	1.9	—	—
Cottonseed	0.5	1.6	—	—
Hydrogenated fat	0.26	0.8	—	—
Calcium carbonate	0.13	0.4	0.03	0.2
Salts	0.12	0.4	0.16	1.1
Nutrient composition	**% DM[2]**	**% WB**	**% DM**	**% WB**
Dry matter	—	52.70	—	63.14
Forages	—	76.80	—	79.00
Concentrates	—	23.20	—	21.00
Crude protein	15.08	7.95	8.98	5.67
Digestible protein	11.44	6.03	5.36	3.38
Fiber	18.96	9.99	27.29	17.23
NDF[3]	39.42.	20.78	57.87	36.54
ADF[4]	21.45	11.31	36.58	23.10
ADL[5]	3.35	1.77	6.85	4.33
Lipid	5.85	3.08	2.90	1.83
Ash	7.52	3.96	8.20	5.18
Starch	21.70	11.44	16.98	10.72
NSC[6]	32.14	16.94	22.05	13.92
Calcium	0.86	0.45	0.45	0.28
Phosphorus	0.44	0.23	0.49	0.31
Magnesium	0.15	0.08	0.20	0.13

[1]WB, wet body.
[2]DM, dry matter.
[3]NDF, neutral detergent fiber.
[4]ADF, acid-detergent fiber.
[5]ADL, acid-detergent lignin.
[6]NSC, nonstructural carbohydrates.

the clinical sign of pathology. Both additive and placebo were mixed with water to obtain a cream. Animals were moved to the barn where there was a corridor, and cream (about 300 mL) was orally administered to each buffalo directly in the mouth using a large syringe as described by Maggiolino et al. (2019).

The trial started 60 d before the expected calving day, up to 60 d in milking, for a total of 120 d. At calving, all animals were moved to different barns (allowing the administration of the lactating buffaloes TMR).

Daily milk yield was recorded and mean daily production of the experimental period was calculated.

Feed composition

TMR samples of both dry and lactating buffaloes were sampled every 20 d and analyzed in triplicate in order to monitor the feed quality. DM was determined using standard procedures (AOAC, 2005; method 930.15). Ash was determined by standard procedures (AOAC, 2005; method 942.05) using a muffle furnace at 550 °C for 16 h. Fat was determined using the Soxhlet extraction procedure (AOAC, 2005; Method 991.36), and crude protein was determined by Kjeldahl N × 6.25 procedures (AOAC, 2005; Method 968.06). Neutral detergent fiber (**NDF**) was determined with the ANKOM fiber analyser according to (Van Soest et al., 1991) and was corrected for residual acid-insoluble ash. Sodium sulfite was added to the solution for NDF determination.

Colostrum and milk sampling and analysis

From each buffalo cow, a colostrum sample was collected within 2 h from calving and Brix degrees were measured

using a refractometer (Pal-Colostrum Pocket refractometer, Bellevue, WA, USA).

A sample of milk from each buffalo cow was collected at +5, +15, +30, +45, and +60 d after calving in 50 mL tubes. Two percent of 2-bromo-2-nitro-1,2-propanediol (Bronopol 98%) was added to each sample as a preservative. Samples were soon cooled at 4 °C and transported to the laboratory and analyzed within 24 h from collection. Samples were analyzed for milk composition (fat [%], protein [%], casein [%], lactose [%], urea [mg/dL], and β-hydroxybutyrate [mmol/L]) using infrared spectroscopy at the milk laboratory of the Breeders Association of Basilicata region (Potenza, Italy) with a MilkoScan 7 RM (FTIR; Foss Electric A/S, Hillerød, Denmark), and somatic cell count (**SCC**) and differential somatic cell count (**DSCC**) were assessed using a Fossomatic 7/7 DC (Foss Electric A/S). The analysis of DSCC is based on the Foss DSCC Method Cell Staining (international patent PCT/EP2010/065615-Holm, 2012) as described by Damm et al. (2017). DSCC is expressed as the combined proportion (%) of polymorphonuclear cells (**PMN**) and lymphocytes on the overall count of milk cells.

Blood sampling and analyses

Blood samples were collected from each buffalo at −60 d (60 d from the expected calving, the day of trial start), −30 (30 d before the expected calving day), 0 (calving day), +15, +30, and +60 d (respectively 15, 30, and 60 d in milking; Figure 1). Blood was aseptically drawn by the lateral coccygeal veins and sinuses using disposable needles (22G), with a negative pressure system for serum (two 9 mL tubes without anticoagulant and with clot activator) and plasma (two 9 mL tubes with 15 USP U/mL of heparin; Becton, Dickinson Canada Inc, Vacutainer, Oakville, Canada). All the tubes were immediately centrifuged in the farm within 5 min after collection (1,500 × *g* for 10 min the plasma and 3,000 × *g* for 10 min the serum). Serum and plasma were then stored at −20 °C until analysis. Moreover, at −60, −30, 0, +15, and +30 d, four 9 mL EDTA tubes were collected for peripheral blood mononuclear cells (PBMCs) analysis, and they were transported at room temperature to the laboratory, no more than 2 h after sampling.

Chemical-biochemical parameters

Clinical biochemistry parameters were obtained from the serum samples using an automated biochemistry analyzer (CS-300B; Dirui, Changchun, China) as reported by De Palo et al. (2018). The following parameters were assessed: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, glucose, creatinine, total protein, albumin, cholesterol, triglycerides, nonesterified fatty acids, calcium, phosphate, magnesium, chloride, bilirubin, urea, uric acid (Gesan Production Kit, Campobello di Mazara, Trapani, Italy). Besides, the concentration of globulins was calculated by subtracting the albumin from the total protein concentration.

Oxidative profile, antioxidant power, and enzymatic activity evaluation

Undiluted plasma was used for the determination of thiobarbituric acid reactive substances (**TBARS**), lipid hydroperoxides, and protein carbonyl according to De Palo et al. (2018). Briefly, a sample (1 mL) was transferred to a glass tube for the TBARS determination and 0.05 mL of butylated hydroxytoluene (7.2% in ethanol) was added along with 1.950 mL of thiobarbituric acid (**TBA**)/trichloroacetic acid (**TCA**)/HCl (0.375% TBA, 15% TCA, and 0.25 N HCl). The sample solution was shaken and then incubated at 90 °C for 15 min in a thermostatic bath. Then, samples were cooled to room temperature (15 to 30 °C) and then centrifuged at 2,000 × *g* for 15 min. Supernatant absorbance at 531 nm was measured against a blank containing 2 mL of TBA/TCA/HCl solution in 1 mL of distilled water. The TBARS were calculated when compared with a standard curve constructed with 1,1,3,3-tetramethoxypropane, and the concentration of lipid oxidation was expressed as milligrams of malondialdehyde (**MDA**) per milliliter of plasma. The intraassay coefficient of variation (CV) was lower than 2.75% and interassay CV was lower than 6.28%.

Protein carbonyls determination was carried out on two aliquots of plasma (50 µL each) that were added with 1 mL 10% TCA and then centrifuged at 1,200 × *g* for 3 min at 4 °C, to measure protein oxidation. One aliquot was used as standard, adding 1 mL of 2 M HCl solution. The second one was added with 1 mL of 2 M HCl containing 10 mM 2,4-dinitrophenyl hydrazine (**DNPH**). The intraassay CV was lower than 3.61% and interassay CV was lower than 5.91%.

Hydroperoxides determination was performed according to De Palo et al. (2013). Briefly, 2 mL of plasma were added with 4 mL of CH_3OH and 2 mL of $CHCl_3$. The samples were vortexed for 30 s and were added with 2 mL of $CHCl_3$ and 1.6 mL of 0.9% NaCl. The samples were shaken for 1 min and then centrifuged at 3,500 × *g* for 10 min at 4 °C. Two milliliters of lipid extract were sampled from the lower chloroform phase and processed with 1 mL of CH_3COOH/$CHCl_3$ and 50 µL of KI (1.2 g/1 mL distilled water). Samples were stored for 5 min in a dark room and added with 3 mL of 0.5% of CH_3COOH and then vortexed and centrifuged at 4,500 × *g* for 10 min at 40 °C. Absorbance at 353 nm was measured against a blank title in which plasma was replaced by 2 mL of distilled water. Results were expressed in µM/mL according to Buege and Aust (1978). The intraassay CV was lower than 3.12% and interassay CV was lower than 6.44%.

Ferric reducing antioxidant power (**FRAP**) assay was calculated according to the method described by Benzie and Strain (1996) with a slight modification as described by Dinardo et al. (2020). Three milliliters of freshly prepared FRAP reagent (1 mL of a 10 m*M* 2,4,6 tripyridyl-*s*-triazine solution in 40 mM HCl plus 1 mL of 20 m*M* $FeCl_3$ and 10 mL of 300 mM acetate buffer, pH 3.6) were incubated at 37 °C for 40 min after mixing with 100 µL of plasma sample or supernatant. The absorbance of the reaction mixture was recorded at 593 nm and the antioxidant power was expressed as micromole Trolox equivalents per mL. The intraassay CV was lower than 3.89% and interassay CV was lower than 4.41%.

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (**ABTS**) radical scavenging activity was assayed by the methods of Re et al. (1999) with some modification. Briefly, ABTS radical cation was produced by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and keeping the mixture in the dark at room temperature for 12 to 16 h before use. For the evaluation of antioxidant capacity, the solution was then diluted in PBS to obtain the absorbance value of 0.70 ± 0.02 at 734 nm. Plasma or supernatant samples (10 µL) were added to 990 µL of diluted ABTS radical cation solution and incubated at

30 °C for 5 min. The reagent blank was prepared by adding 10 µL of PBS instead of the sample. Then, the scavenging of the ABTS radical cation was determined spectrophotometrically at 734 nm. Antioxidant activity was expressed as a percentage inhibition (I) of ABTS radical cation and calculated by the equation.

$$\%\,\text{inhibition} = 100 \times (\text{Absorbance}_{734}\ \text{control}\ \text{Absorbance}_{734}\ \text{sample}) / \text{Absorbance}_{734}\ \text{control}$$

The intraassay CV was lower than 2.41% and the interassay CV was lower than 5.69%.

Superoxide dismutase (**SOD**, EC 1.15.1.1), catalase (**CAT**, EC 1.11.1.6), and glutathione peroxidase (**GPx**, EC1.11.1.9.) activities were measured and calculated as described by Maggiolino et al. (2021). For SOD, the intraassay CV was lower than 2.68% and the interassay CV was lower than 4.41%; for CAT, the intraassay CV was lower than 2.96% and the interassay CV was lower than 5.98%; for GPx, the intraassay CV was lower than 3.86% and the interassay CV was lower than 5.29%.

Determination of acute phase proteins and interleukins

The plasma samples were used for determination of C-reactive protein (CRP), haptoglobin (Hp), interleukin (IL)-1α, IL-1β, IL-8 IL-10 (Cloud-Clone corporation, Katy, TX, USA), TNF-α, IL-2 (Mybiosource, The Netherlands), IL-4, and IL-6 (Alpha Diagnostic, San Antonio, TX, USA), according to the manufacturer's instructions and using an automated microplate ELISA reader (Tecan Detection Infinite200 Pro). The plates were read at 450 nm. Values were expressed in mg/L (Hp), ng/L (CRP, IL-8), and pg/mL (TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-6, and IL-10). All assays involve bovine antibodies and were validated by Smith et al. (2021). Enzyme-linked immunosorbent assay components, buffalo plasma dilutions, minimum detectable, detection range, intra and inter assay CV, and manufacturers are reported in Table 2. All samples were tested in duplicate. These analyses were performed at −30, 0, 15, 30, and 60 d.

Isolation of PBMCs and monocytes

PBMCs were isolated from peripheral blood using a Ficoll–Hypaque gradient according to Latronico et al. (2007). Briefly, 20 mL of whole blood, diluted 1:1 with cold PBS, was layered on 10 mL of Histopaque-1077 solution and centrifuged at 400 × *g* for 30 min at 20 °C. The white cell rings were recovered, washed twice with PBS, and then pellets were suspended in Iscove's Modified Dulbecco's medium (**IMDM**), without calcium and magnesium (Sigma Aldrich, Milan, Italy). Cells were counted in a Burker cell counting chamber and the viability of cells was assessed by Trypan blue (Sigma Aldrich, Milan, Italy) dye exclusion. For the isolation of monocytes, PBMC suspension in IMDM/10% FCS was plated in 96-well flat-bottom microplates (100 µL, 2×10^6 cells/well). After incubation for 1 h at 37 °C in 5% CO_2, nonadherent lymphocytes were collected and adherent cells, represented by monocytes (about 2×10^5 cells/well), were washed several times. Samples of PBMC suspension (100 µL, 2×10^5 cells/well) were plated in 96-well U-bottom microplates in serum-free medium and treated with concanavalin A (**ConA**, at final concentration of 5 µg/mL) or hydrogen peroxide ($\mathbf{H_2O_2}$ at a final concentration of 1 mM) and cultured at 37 °C in 5% CO_2. Non-treated PBMCs represented the negative control (**CTRL**). After incubation for 20 h, the plates were centrifuged at 400 × *g*, 10 min, 20 °C in a microplate centrifuge, then the culture medium was collected, centrifuged at 10,000 *g* and supernatants were stored at −80 °C until analysis.

Reactive oxygen species and reactive nitrogen species detection in PBMCs and monocytes

Intracellular free radicals concentration was carried out by loading PBMCs (2×10^5 cells/well in 96-well plates) or monocytes (2×10^5 cells/well in 96-well plates) with 10 µM 20,70-dichlorofluorescein diacetate (**DCFH-DA**) in phenol red-free IMDM at 37 °C for 30 min (Latronico et al., 2021), then treated for 1 h and 30 min with ConA, at final concentration of 5 µg/mL, or H_2O_2 at a final concentration of 1 mM. After incubation, the culture medium was removed, and cells were rinsed twice with PBS. Cells were resuspended in phenol

Table 2. Enzyme-linked immunosorbent assay components, buffalo plasma dilutions, minimum detectable, detection range, intra and inter assay CV (expressed as %), and manufacturers

Analyte	Plasma dilution[1]	Detection range, pg/mL	Minimum detectable, pg/mL	Intraassay CV[2]	Interassay CV	Manufacturer
Bovine IL-1α	1:2	15.6 to 1,000	5.9	<2.56	<6.32	Cloud-Clone corporation
Bovine IL-1β	1:2	15.6 to 1,000	6.5	<3.33	<4.23	Cloud-Clone corporation
Bovine IL-2	1:2	15.6 to 1,000	6.4	<4.21	<7.28	Mybiosource
Bovine IL-4	1:2	15.6 to 1,000	9.37	<3.95	<5.92	Alpha Diagnostic
Bovine IL-6	1:1	31.25 to 2,000	18.75	<2.86	<8.12	Alpha Diagnostic
Bovine IL-8	1:10	15.6 to 1,000	6.2	<3.19	<7.39	Cloud-Clone corporation
Bovine IL-10	1:1,000	15.6 to 1,000	6.4	<2.76	<5.49	Cloud-Clone corporation
Bovine CRP	1:1,000	6.25 to 400	2.43	<2.51	<6.19	Cloud-Clone corporation
Bovine Hp	1:4,000	15.6 to 1,000	5.9	<2.65	<6.74	Cloud-Clone corporation
Bovine TNF-α	1:1	15.6 to 1,000	5	<3.92	<8.91	Mybiosource

[1]Final plasma dilution in each assay.
[2]Coefficient of variation.
IL-1α, interleukin 1 alpha; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; CRP, C-reactive protein; Hp, haptoglobin; TNF-α, tumor necrosis factor alpha.

red-free IMDM and the spectrofluorometric analysis was performed at 485 nm excitation/525 nm emission. Negative control was represented by cells treated with the same experimental protocol with DCFH-DA. reactive oxygen species (ROS) production was expressed as a relative percentage of photoluminescence (**PL**) intensity with respect to the negative control.

Relative levels of ROS in PBMC supernatants were measured by OxiSelectTM in Vitro **ROS**/reactive nitrogen species (**RNS**) Assay Kit Green Fluorescence (Cell Biolabs Inc, ell Biolabs Inc., San Diego, CA) according to manufacturer's instructions. This assay is based on the reaction of ROS and RNS species with DCFH, which is rapidly oxidized to the highly fluorescent 2′,7′-dichlorodihydrofluorescein (**DCF**). Green fluorescence was read with a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 480 nm excitation /530 nm emission. The fluorescence of blank samples was subtracted from sample measurements to eliminate background fluorescence. The fluorescence intensity was directly proportional to the total ROS/RNS levels within the sample and was expressed as IF arbitrary units.

Proliferation assay and viability

Cell viability or cytotoxicity of PBMCs from the different groups of animals was detected by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (**MTT**) assay as described by Di Bari et al. (2014). This assay is based on the reduction of MTT by the mitochondrial succinate dehydrogenase in viable cells, to a blue formazan product which can be spectrophotometrically measured by using a microplate reader. This assay was specifically identified to detect cell viability and proliferation since, differently from other colorimetric or fluorescent dyes used to detect cell viability in cultured cells, it allows to distinguish between healthy cells and cells that, though still alive, already lost their vital functions.

Briefly, after the removal of the culture medium, cells were rinsed with PBS and incubated at 37 °C, 5% CO_2 for 2 h with 0.5 mg/mL MTT. After centrifugation of the microplates, the reaction was stopped by removing the medium and the formazan crystals in the cells were solubilized with absolute ethanol. The absorbance values at 560 and 690 nm were recorded by means of a VersaMax Microplate Reader (Molecular Devices). The difference between the absorbance of each sample at 560 and 690 nm was calculated. The value of the untreated samples of the different groups of animals (CTRL) was set at 100% and the cell viability was expressed as percentage of CTRL.

Statistical analysis

Different data sets were considered and subjected to different statistical analyses. Each animal represented an experimental unit. All data sets were tested for normal distribution (Shapiro–Wilk) and variance homogeneity (Bartlett test). In order to cope with non-normality, SCC was log-transformed into somatic cell score (**SCS**) using the formula proposed by Ali and Shook (1980):

$$\mathrm{SCS} = \log_2\left(\frac{\mathrm{SCC}}{100,000}\right) + 3.$$

DSCC was log-transformed into differential somatic cell score (**DSCS**) using the formula reported by Ablondi et al. (2023):

$$\mathrm{DSCS} = \log_2\left[\left(\frac{\mathrm{DSCC}}{100}\right) \times \mathrm{SCC} \times 10^{-5}\right] + 3.$$

Moreover, IL-1α, IL-1β, IL-2, IL-4, IL-6, and IL-10 data were log-transformed for statistical analysis and then reported at normal value, as they showed to be not normally distributed in the raw dataset.

Biochemical-clinical, electrolytic, oxidative, and antioxidant parameters were subjected to analysis of variance (**ANOVA**) according to the General Linear Model (**GLM**) procedure as reported in the following model:

$$y_{ijk} = \mu + \alpha_i + D_j + T_k + (D \times T)_{jk} + \varepsilon_{ijkl},$$

where y_{ijk} represents all blood variables; μ is the overall mean; α_i is the constant of the individual buffalo random effect (i = 1,…,80); D represents the effect of the jth oral additive administration treatment (j = 1, 2), T was the effect of the kth time (k = 1,…,6), $D \times T$ represents the binary interaction between the jth oral additive administration and the kth time (1,…,12).

After a first analysis, differences were observed between primiparous and multiparous data for SCS and DSCS. For this reason, data were subdivided into two sub datasets, one from primiparous and one from multiparous buffalo cows. After this, inflammatory markers, milk parameters, SCS (primiparous and multiparous separated), and DSCS (primiparous and multiparous separated) were subjected to ANOVA according to the GLM procedure as reported in the following model:

$$y_{ijk} = \mu + \alpha_i + D_j + T_k + (D \times T)_{jk} + \varepsilon_{ijkl},$$

where y_{ijk} represents all dependent variables; μ is the overall mean; α_i is the constant of the buffalo random effect (i = 1,…,80); D was the effect of the jth oral additive administration treatment (j = 1, 2), T was the effect of the kth time (k = 1,…,5), $D \times T$ represents the binary interaction between the jth oral additive administration and the kth time (1,…,10). A Tukey test was applied to evaluate the differences among means when the effect of time or the binary interaction of treatment × time was significant. Pairwise comparison was performed by Bonferroni test.

Differences were observed between primiparous and multiparous data for average milk yield. For this reason, data were subdivided into two sub datasets, one from primiparous and one from multiparous buffalo cows. Data on daily average milk yield (kg/head/d) were subjected to ANOVA as reported by the following model:

$$y_{ij} = \mu + \alpha_i + D_j + \varepsilon_{ijk},$$

where y_{ij} are dependent variables; μ is the overall mean; α_i is the constant of the buffalo random effect (i = 1,…,80); D represents the effect of the jth oral additive administration treatment (j = 1, 2), and ε_{ijk} was the error term.

No differences between parities were observed in brix degrees, cell viability, and ROS within each experimental time.

These data were subjected to one-way ANOVA as described by the following model:

$$y_{ijk} = \mu + \alpha_i + D_j + \varepsilon_{ijk},$$

where Y_{ijk} represents the dependent variables, μ is the overall mean, α_i is the ith buffalo random effect (i = 1,…,80), D represents the effect of the jth oral additive administration treatment (j = 1, 2), and ε_{ijk} is the error term. Pairwise comparison was performed by Bonferroni test.

Significance was set at $P < 0.05$, and the results were expressed as means and mean standard error. All the analyses were performed using SAS software (SAS, 2018).

Results

Milk yield, chemical composition, SCS, DSCS, and colostrum brix degrees

Milk yield was not affected by the dietary treatment both in primiparous and multiparous buffaloes' cows (Figure 2), although a little tendence to an increase ($P < 0.10$) in milk yield was observed in DEC buffaloes' cows in both primiparous and multiparous.

The effects of days in milk (**DIM**) and of the dietary treatment are reported in Table 3. All investigated milk parameters were affected by DIM ($P < 0.001$), but no effects of dietary treatment or the binary interaction were observed ($P > 0.05$). DM, protein, casein, and fat concentration decreased at 15th DIM ($P < 0.01$) and then remained at constant values up to 60 DIM in both experimental groups. Lactose concentration, instead, showed a steady increase up to 45 DIM in both groups ($P < 0.01$) and then did not vary until 60 DIM.

The SCS and DSCS values are reported in Figure 3. Both these patterns were shown to be affected by time in primiparous buffalo cows ($P < 0.001$) and by time, treatment, and their binary interaction in multiparous buffalo cows ($P < 0.001$). The SCS showed a decreasing trend during the experimental trial in primiparous buffalo cows (Figure 3a), with lower values at 60 DIM compared to 5 ($P < 0.01$) and 15 ($P < 0.05$) DIM in the CON group. Lower values of SCS were detected at 60 DIM compared to 5 DIM ($P < 0.05$) in DEC group. Multiparous Buffaloes (Figure 3b) from CON group showed a decreasing trend up to 30 DIM, with lower values compared to 5 DIM ($P < 0.01$). Those of DEC group had a similar decreasing trend with lower values after 15 d ($P < 0.01$) and then remained almost constant. Moreover, SCS values of DEC buffaloes were lower than CON in the first 15 DIM ($P < 0.01$). The DSCS in primiparous was affected only by time ($P < 0.01$) in CON group, reporting at 60 DIM lower values than 5 ($P < 0.01$) and 15 ($P < 0.05$) DIM. On the other hand, DSCS values in DEC group did not vary during the experimental period ($P > 0.05$). Multiparous buffalo cows showed a decreasing trend in both groups, with lower values at 15 and 30 DIM in CON animals ($P < 0.01$) and lower values at 15 DIM in DEC animals ($P < 0.05$) compared to 5 DIM; after they remained constant until 60 DIM.

Colostrum brix degrees values were not affected by the dietary treatment, showing similar values ($P > 0.05$).

Blood oxidative and inflammatory profile, cell viability, ROS production, and ROS/RNS ratio

Oxidative profile of buffalo cows is reported in Table 4. The ABTS, FRAP, SOD, CAT, and GSPx showed to be affected by dietary treatment, time, and their binary interaction ($P < 0.01$). Differently, protein carbonyls, hydroperoxides and TBARS did not show any variation ($P > 0.05$).

The ABTS values increased constantly in both groups from −60 d up to calving day ($P < 0.01$), then decreased until 30 DIM in CON group ($P < 0.01$) and at 15 DIM ($P < 0.01$) and 60 DIM ($P < 0.05$) in DEC group. Animals from the DEC group recorded higher ABTS values from -30d to 60 DIM than CON ones ($P < 0.01$). The FRAP values increased at calving day in CON group ($P < 0.01$) and then decreased at 15 DIM ($P < 0.01$) and 60 DIM ($P < 001$) reaching values lower than those registered before calving. Differently, in DEC group the FRAP values increased at −15 d ($P < 0.01$) and then remained constant until 60 DIM. Moreover, from −15 d to 60 DIM, the FRAP values recorded in DEC group were higher than those in CON group ($P < 0.01$). In control group animals, we detected higher activity of SOD at calving

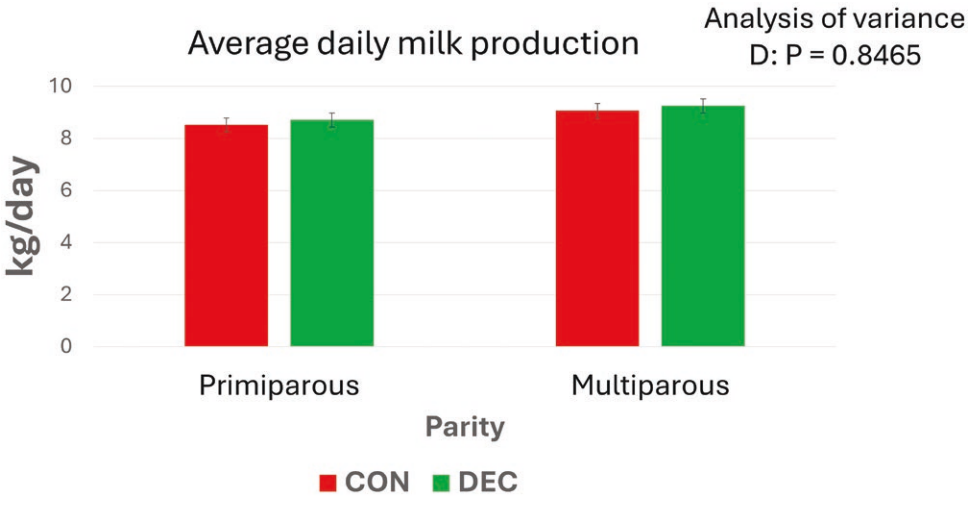

Figure 2. Average daily milk production in buffaloes treated with dietary supplementation of Decosel (DEC) and untreated buffaloes (CON) expressed as kg/d ± SD. D, oral additive administration effect; T, time effect.

Table 3. Acetone, β-hydroxybutyrate (BHB), urea, dry matter (DM), lactose, protein, casein, and fat quantification in milk of buffaloes treated with dietary supplementation of Decosel (DEC) and untreated buffaloes (CON) during first 60 d of milking

Parameters	Group	Time					SEM	*P*-value		
		+5	+15	+30	+45	+60		G[1]	T[1]	G × T[1]
DM, %	CON	20.88^{A}	17.95^{B}	17.54^{B}	17.40^{B}	17.60^{B}	0.33	0.964	<0.001	0.648
	DEC	20.57^{A}	17.98^{B}	17.35^{B}	17.27^{Ba}	18.24^{Bb}				
Protein, %	CON	5.60^{A}	4.73^{Ba}	4.56^{B}	4.50^{Bb}	4.52^{B}	0.08	0.272	<0.001	0.812
	DEC	5.41^{A}	4.73^{Ba}	4.50^{B}	4.48^{Bb}	4.50^{B}				
Casein, %	CON	4.44^{A}	3.73^{B}	3.62^{B}	3.58^{B}	3.60^{B}	0.07	0.382	<0.001	0.793
	DEC	4.28^{A}	3.74^{B}	3.57^{B}	3.57^{B}	3.60^{B}				
Fat, %	CON	9.89^{A}	7.55^{B}	7.10^{B}	7.80^{B}	7.00^{B}	0.28	0.935	<0.001	0.738
	DEC	9.77^{A}	7.44^{B}	7.91^{B}	7.68^{Ba}	7.49^{Bb}				
Lactose, %	CON	3.80^{A}	4.52^{Ba}	4.76^{BCb}	5.02^{D}	4.94^{CD}	0.07	0.051	<0.001	0.852
	DEC	3.88^{A}	4.64^{B}	4.84^{BC}	5.03^{CD}	5.10^{D}				
Urea, mg/dL	CON	41.64^{ab}	37.60^{a}	39.21^{ab}	40.93^{ab}	42.34^{b}	1.70	0.822	0.002	0.884
	DEC	40.86^{AB}	35.78^{A}	38.88^{AB}	40.97^{AB}	44.05^{B}				
BHB, mM	CON	0.13^{Aa}	0.20^{ABb}	0.26^{Bc}	0.20^{ABb}	0.22^{Bc}	0.02	0.900	<0.001	0.705
	DEC	0.15^{Aa}	0.21^{AB}	0.24^{B}	0.23^{AB}	0.21^{ABb}				

[1]G, group; T, time; G × T, group × time.
Different letters on the same line show statistical differences during time in the same group: $^{A,B,C}P < 0.01$; $^{a,b,c} = P < 0.05$.

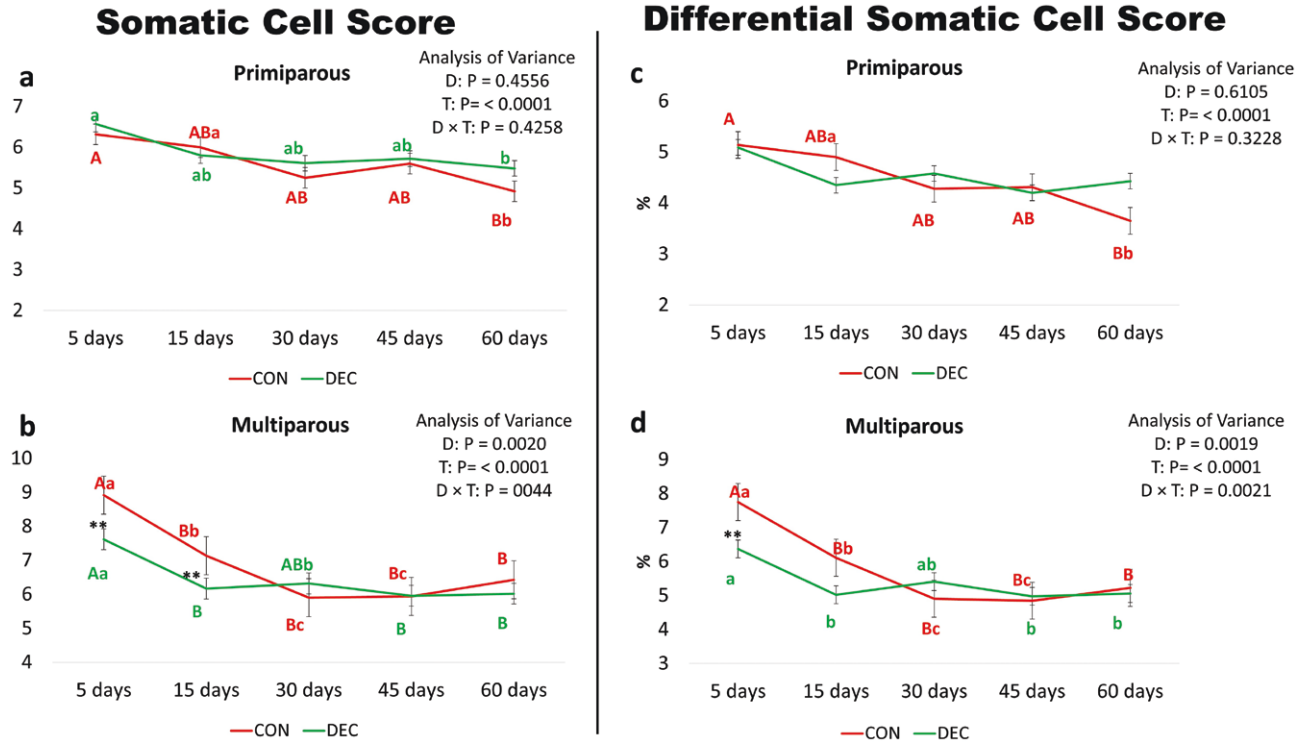

Figure 3. Somatic cell score (SCS) and differential somatic cell score (DSCS) in primiparous (a and b) and multiparous (c and d) buffaloes treated with dietary supplementation of Decosel (DEC) and untreated buffaloes (CON) during 60 d (5, 15, 30, 45, and 60 d in milk) of lactation. Different letters of the same color show statistical differences among experimental times in each experimental groups: $^{A,B}P < 0.01$; $^{a,b,c}P < 0.05$. **Statistical differences between groups at the same experimental time: $^{**}P < 0.01$. D, oral additive administration effect.

day ($P < 0.01$); then they decreased at 15 DIM ($P < 0.01$) and did not vary until the end of the trial. Differently, in DEC group, the SOD activity increased at calving ($P < 0.01$) and then increased again at 30 DIM ($P < 0.01$). Moreover, the SOD values in DEC animals were higher than CON animals from −30 d to 60 DIM ($P < 0.01$). CAT activity in CON buffalo cows increased at calving ($P < 0.01$) and decreased at 30 DIM ($P < 0.01$), while in DEC group increased constantly during the trial ($P < 0.01$) with higher values than CON group from −30 to +60 d ($P < 0.01$). CON group showed an increase in GSPx activity at calving day ($P < 0.01$) with a decrease at 30 DIM ($P < 0.05$). The DEC group showed a

Table 4. Antioxidant potential and oxidation products in plasma of buffaloes treated with dietary supplementation of Decosel (DEC) and untreated buffaloes (CON) from 60 d before calving to 60 d in milking

Parameter	Group	Time						SEM	*P*-value		
		−60	−30	0	+15	+30	+60		G[1]	T[1]	G × T[1]
ABTS, % I	CON	29.84^{A}	32.17^{BX}	36.74^{CX}	34.12^{DX}	33.40^{BCX}	33.24^{BC}	0.49	<0.001	<0.001	<0.001
	DEC	28.54^{A}	35.68^{BY}	40.35^{CY}	36.12^{BaY}	35.96^{BaY}	34.36^{Bb}				
FRAP, µmol TE/mL	CON	35.74^{A}	36.04^{AX}	38.56^{Bx}	29.23^{CX}	29.40^{CX}	27.18^{DX}	0.55	<0.001	<0.001	<0.001
	DEC	35.51^{A}	38.54^{BaY}	40.32^{Bby}	39.40^{BY}	39.84^{BY}	39.38^{BY}				
SOD, U/mL	CON	52.91^{A}	55.97^{AX}	75.11^{BX}	63.82^{CX}	63.61^{CX}	63.44^{CX}	1.14	<0.001	<0.001	<0.001
	DEC	52.12^{A}	102.48^{BaY}	105.86^{BCbY}	108.86^{CY}	125.50^{DY}	125.82^{DY}				
Cat, U/mL	CON	3.51^{A}	3.74^{AX}	4.52^{BX}	4.46^{BX}	4.09^{CX}	4.00^{CX}	0.87	<0.001	<0.001	<0.001
	DEC	3.31^{A}	6.17^{BY}	7.67^{CY}	8.45^{DY}	8.87^{EY}	9.21^{FY}				
GSPx, nmol NADPH ox/min/mL	CON	37.32^{A}	37.07^{AX}	45.08^{BaX}	43.99^{BX}	43.85^{BX}	43.24^{BbX}	0.58	<0.001	<0.001	<0.001
	DEC	36.33^{A}	50.85^{BY}	56.40^{CY}	57.91^{CDY}	59.92^{EY}	61.58^{EY}				
Hydroperoxides, mmol/mL	CON	5.68	6.15	5.95	6.08	5.75	5.96	0.11	0.220	0.054	0.185
	DEC	5.93	6.19	6.01	5.99	6.13	5.81				
TBARS, mmol/mL	CON	0.88	0.88	0.89	0.85	0.84	0.81	0.03	0.217	0.107	0.463
	DEC	0.82	0.91	0.96	0.85	0.91	0.85				
Protein Carbonyls, µmol/mg Protein	CON	99.32	99.45	101.33	102.33	98.29	100.79	4.58	0.251	0.551	0.485
	DEC	99.45	99.43	102.71	100.80	101.13	100.52				

[1]G, group; T, time; G × T, group × time.
Different letters on the same line show statistical differences during time in the same group: $^{A,B,C,D,E,F}P < 0.01$; $^{a,b}P < 0.05$. Different letters on the same row show statistical differences between groups at the same time: $^{X,Y}P < 0.01$; $^{x,y}P < 0.05$.

constant increase from −30 d ($P < 0.01$) to 60 DIM, reporting always higher values then the CON one at the same experimental times ($P < 0.01$). The highest protein carbonyl concentrations in CON group were registered at calving day and at 15 DIM (P < 0.01), while the highest in DEC group were observed only at calving day ($P < 0.01$).

The inflammatory profile results are reported in Table 5. Time affected IL-1α, IL-6, IL-8, CRP, Hp, and TNF-α ($P < 0.01$). Concentration levels of IL-1α decreased at 60 DIM in both groups ($P < 0.01$), as well as those of IL-6 decreased at 60 DIM both in CON ($P < 0.05$) and DEC ($P < 0.01$) groups. The IL-8 concentration levels of CON group increased at +30 d ($P < 0.01$) and then decreased at +60 d ($P < 0.01$). Differently, in DEC group, this interleukin decreased at 60 DIM ($P < 0.01$). The CRP plasma concentration in CON buffaloes increased at 15 DIM ($P < 0.01$), then decreased at 60 DIM ($P < 0.01$), while in DEC group raised at calving and at 15 DIM ($P < 0.01$) and then dropped at 60 DIM ($P < 0.01$). The Hp values increased in both groups at calving ($P < 0.01$) and then decreased at 60 DIM ($P < 0.01$) and 15 DIM ($P < 0.05$) in CON and DEC animals, respectively. TNF-α concentration at 60 DIM was lower than 15 DIM and 30 DIM in CON group ($P < 0.01$). Consistently, in DEC group, TNF-α peaked at 60 DIM ($P < 0.01$).

PBMC's cell viability results are shown in Figure 4. There were no differences according to the dietary treatment at −60 and −30 d in PBMCs cell viability in control group and after challenge with ConA and H_2O_2 ($P > 0.05$). At calving, DEC animals showed higher PBMC viability than the CON group, also after stimulation with ConA and H_2O_2 ($P < 0.01$). At 15 DIM and 30 DIM, cell viability values were higher in DEC only after ConA stimulation ($P < 0.01$).

Figure 5 shows the PBMCs' ROS production results in the intracellular district. DEC animals showed lower ROS concentration than CON group at −30 d and 15 DIM after H_2O_2 stimulation ($P < 0.0001$), as well as at calving day, at 30 DIM without stimulation ($P < 0.01$) and after H_2O_2 stimulation ($P < 0.0001$).

PBMCs' ROS/RNS ratio intensity in the culture medium is reported in Figure 6. This pattern was affected by the experimental group, time, and their binary interaction ($P < 0.01$). The CON group showed an increased ROS/RNS ratio at calving day ($P < 0.01$) and then a decrease at 30 DIM ($P < 0.01$). Differently, DEC group showed at −30 d lower values than −60 d and calving day ($P < 0.01$). After calving these values decreased again at 15 DIM ($P < 0.05$) and 30 DIM ($P < 0.01$). CON group showed higher ROS/RNS values than DEC group at −30 d and at calving day ($P < 0.01$).

Discussion

The peripartum period, also known as the transition period, is the most critical for the immediate and long-term performances of dairy ruminants. Several strategies were suggested to cope with the negative impacts of the transition period in ruminants, including dietary additive supplementation (Cherif et al., 2018). Over the last decade, the administration of feed additives during the peripartum has gained the interest of researchers and producers. Although some studies have been conducted on the effect of hydrolyzed yeast (Humer et al., 2018; Stefenoni et al., 2020) or yeast walls (Aung et al., 2019) on health status and performances during the transition period in dairy cows, no studies have been conducted on buffaloes, as well as on DE effects in ruminants during the peripartum period. Thus, our trial focused on the effects of oral administration of a commercial mix (Decosel) of yeast walls, hydrolyzed yeasts and DE during peripartum on the

Table 5. Inflammatory markers in buffaloes treated with dietary supplementation of Decosel (DEC) and untreated buffaloes (CON) from 30 d before calving to 60 d in milking

Parameter	Group	Time					SEM	*P*-value		
		−30	0	+15	+30	+60		G[1]	T[1]	G × T[1]
IL-1α, pg/mL	CON	472.00^{A}	494.24^{A}	541.74^{A}	511.55^{A}	307.69^{B}	36.60	0.074	0.001	0.485
	DEC	575.84^{A}	540.18^{A}	526.74^{A}	529.11^{A}	384.93^{B}				
IL-1β, pg/mL	CON	506.62	558.29	554.12	511.54	493.54	32.04	0.223	0.271	0.701
	DEC	578.37	586.87	539.16	524.08	515.83				
IL-2, pg/mL	CON	625.42	669.21	709.50	697.04	687.56	22.85	0.755	0.137	0.085
	DEC	667.88	718.50	675.04	643.42	717.63				
IL-4, pg/mL	CON	386.66	417.69	386.90	313.41	289.68	44.20	0.688	0.161	0.451
	DEC	455.10	344.39	380.15	331.24	368.98				
IL-6, pg/mL	CON	243.72^{a}	257.82^{a}	218.10	200.10	156.62^{b}	22.70	0.723	0.018	0.355
	DEC	247.46^{A}	227.22^{A}	212.79^{A}	257.68^{A}	150.32^{B}				
IL-8, ng/mL	CON	4.18^{A}	4.76^{A}	4.34^{A}	5.25^{B}	2.93^{C}	0.29	0.908	<0.001	0.544
	DEC	4.30^{A}	4.61^{A}	4.70^{A}	4.71^{A}	3.13^{B}				
IL-10, ng/mL	CON	231.53	222.32	253.27	226.13	213.86	19.58	0.257	0.760	0.657
	DEC	262.56	236.12	239.97	231.68	258.21				
CRP, ng/mL	CON	198.68^{A}	239.54^{A}	343.51^{B}	361.35^{B}	168.27^{A}	24.89	0.608	<0.001	0.558
	DEC	201.04^{A}	285.95^{B}	361.12^{C}	331.10^{C}	156.16^{A}				
Hp, μg/L	CON	2.73^{A}	3.71^{B}	3.78^{B}	3.12	2.80^{A}	0.38	0.143	0.007	0.821
	DEC	2.34^{A}	3.98^{Ba}	3.24^{b}	3.02^{b}	3.27^{b}				
TNF-α, pg/mL	CON	93.13	114.66	123.41^{A}	113.92^{A}	76.13^{B}	8.91	0.339	<0.001	0.393
	DEC	113.06^{A}	118.49^{A}	112.30^{A}	126.49^{A}	72.23^{B}				

[1]G, group; T, time; G × T, group × time.
IL-1α: interleukin 1 alpha; IL-1β: interleukin 1 beta; IL-2: interleukin 2; IL-4: interleukin 4; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; CRP: C-reactive protein; Hp: haptoglobin; TNF-α: tumor necrosis factor alpha.
Different letters on the same line show statistical differences during time in the same group: $^{A,B,C}P < 0.01$; $^{a,b,c}P < 0.05$.

immune and biochemical status and production in transition dairy buffalo cows.

The Decosel additive administration did not affect milk yield and composition during the early lactation period. Some authors have reported that yeast products administered to cows during the transition period are able to increase milk yield, and sometimes improve also fat and protein content (Nocek et al., 2011; Faccio-Demarco et al., 2019), while results reported by other authors supported our findings, with no effects also on milk yield (Yuan et al., 2015; Wu et al., 2019). Probably, the relatively low number of animals involved and their rather low productive level did not allow a real effect of the additive on production to emerge. It is hypothesized that such effects could more easily emerge in animals with higher levels of production. However, milk production patterns (protein, casein, fat, and lactose percentage, as well as the total DM) were strongly affected by DIM. All our results were consistent with previous findings, falling within the same range (Han et al., 2007; Garau et al., 2021). DM, protein, casein, and fat percentage tended to decrease in the first days in milking, with the increasing of milk yield, while lactose increased. This shows an opposite trend of lactose and protein contents in buffalo milk, especially during the first 15 d when the transition from colostrum to milk occurs. Lactose is the restricting factor in milk secretion in the mammary gland and is actively involved in regulating the osmotic pressure in the epithelial cell of the mammary gland, thus ultimately influencing protein yield (Wang et al., 2019). Additionally, there were no differences in immunoglobulin concentration observed in colostrum between the groups. The measurement of colostrum density and degrees Brix in cows enables the prediction of its immunoglobulin levels with a correlation higher than 95% (Gamsjäger et al., 2020). Colostrum is characterized by high DM content and contains nutrients and bioactive substances, both important for several biological benefits for the newborn calf as energy and passive immunity transfer (McGrath et al., 2016). Although researchers have reported that feeding yeast products can modulate immune functions in both experimental animal models and livestock species (Nocek et al., 2011; El-Naggar and Attia, 2015), similar to our results, no effect of these additives was reported on immunoglobulin colostrum concentration in cows (Yuan et al., 2015; Wu et al., 2019) and goats (Moreno-Indias et al., 2012). The SCC represents the current most simple, practical, and sustainable method to monitor udder health in dairy herds, even if it does not have the same accuracy as microbiological analysis (Alhussien and Dang, 2018; Zecconi et al., 2020). It can only suggest the presence of inflammation, but not the presence of a pathogen. However, there is a scientific consensus worldwide on the association between an increase in SCC in milk and a change in the proportion of inflammatory cells in the multicellular population. It has been suggested that the quantity of polymorphonuclear leukocytes, lymphocytes, and macrophages may be a more useful indicator in the evaluation of udder health than SCC (Stocco et al., 2020). Our results on SCC and DSCS are supported by literature (Boselli et al., 2020; Poudel et al., 2021), considering also that buffaloes are characterized by high SCC variability and that they tend to increase, as our

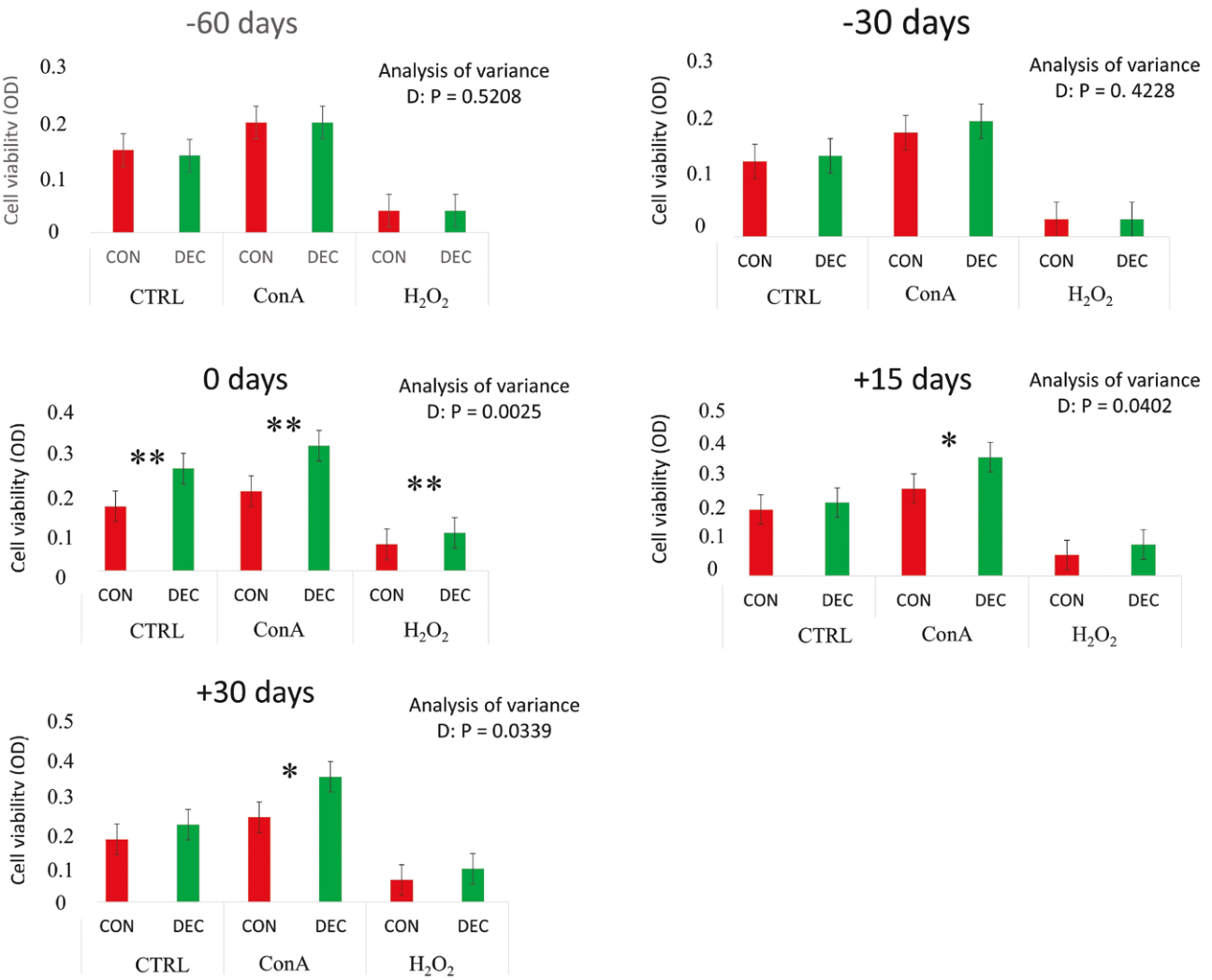

Figure 4. Effect of treatments on cell viability of peripheral blood mononuclear cells (PBMCs) from control group (CON) and Decosel group (DEC) buffaloes during trial. Cell viability, expressed as optical density (OD), was detected by the in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test in untreated PBMCs (negative) or PBMCs treated for 20 h at 37 °C, 5% CO_2 with LPS, concanavalin A (ConA), or H_2O_2. Data are reported as LSM ± SEM. Differences between groups: *$P < 0.05$, **$P < 0.01$. D, oral additive administration effect.

results showed, with age and parity (Fagiolo and Lai, 2007). SCC and DSCS tended to decrease in both primiparous and multiparous buffaloes during the early lactation, when lactation curve reaches the peak (Singh and Ludri, 2001), although oral administration of Decosel seems to be able to reduce both these parameters in the first 15 d in milking only in multiparous ones. It is clear that there was a dilution effect due to DIM, but despite the differences highlighted between the two groups, these did not seem to be able to influence the productive characteristics and quality of the animals. However, an increase in SCC is associated with an increase in DSCS, as expected (Koess and Hamann, 2008). Although the relationship between the increase in SCS and DSCS, and the onset of intramammary infections is still a topic of discussion in this species today, values observed are not linked to clinically evident mammary diseases (Bobbo et al., 2023) during the experimental trial. Studying the effects of yeast product dietary supplementation in early lactation dairy cows, Wu et al. (2019) reported a reduction in SCS and DSCS values in multiparous, consistent with our results. It may be hypothesized that the immuno-modulatory and protective effect of Decosel toward the mammary parenchyma is greater in animals that are more exploited from a productive point of view, such as multiparous buffaloes, and that mammary glands are more susceptible to diseases with more lactations, leading to the improvement of immune function and the activation of the cell-mediated immune response (Chawla and Kaur, 2005).

Total antioxidant capacity and enzyme activity were enhanced by Decosel administration, as early as the precalving period, while the oxidation products did not show differences during the experimental trial. SOD and CAT are major intracellular enzymatic antioxidant systems, considered as the first defense against pro-oxidants. SOD converts the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), which is further converted into less dangerous forms by CAT and other antioxidants (Fee et al., 1975; Bernabucci et al., 2002). GSPx acts later than the previous ones and is able to modulate biochemical pathway that catalyzes the synthesis of prostaglandins, leukotrienes, prostacyclines, and thromboxanes; this is reported to be related to the normal function of the immune system (Halliwell and Gutteridge, 2015). It is a major endogenous antioxidant produced by cells, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such

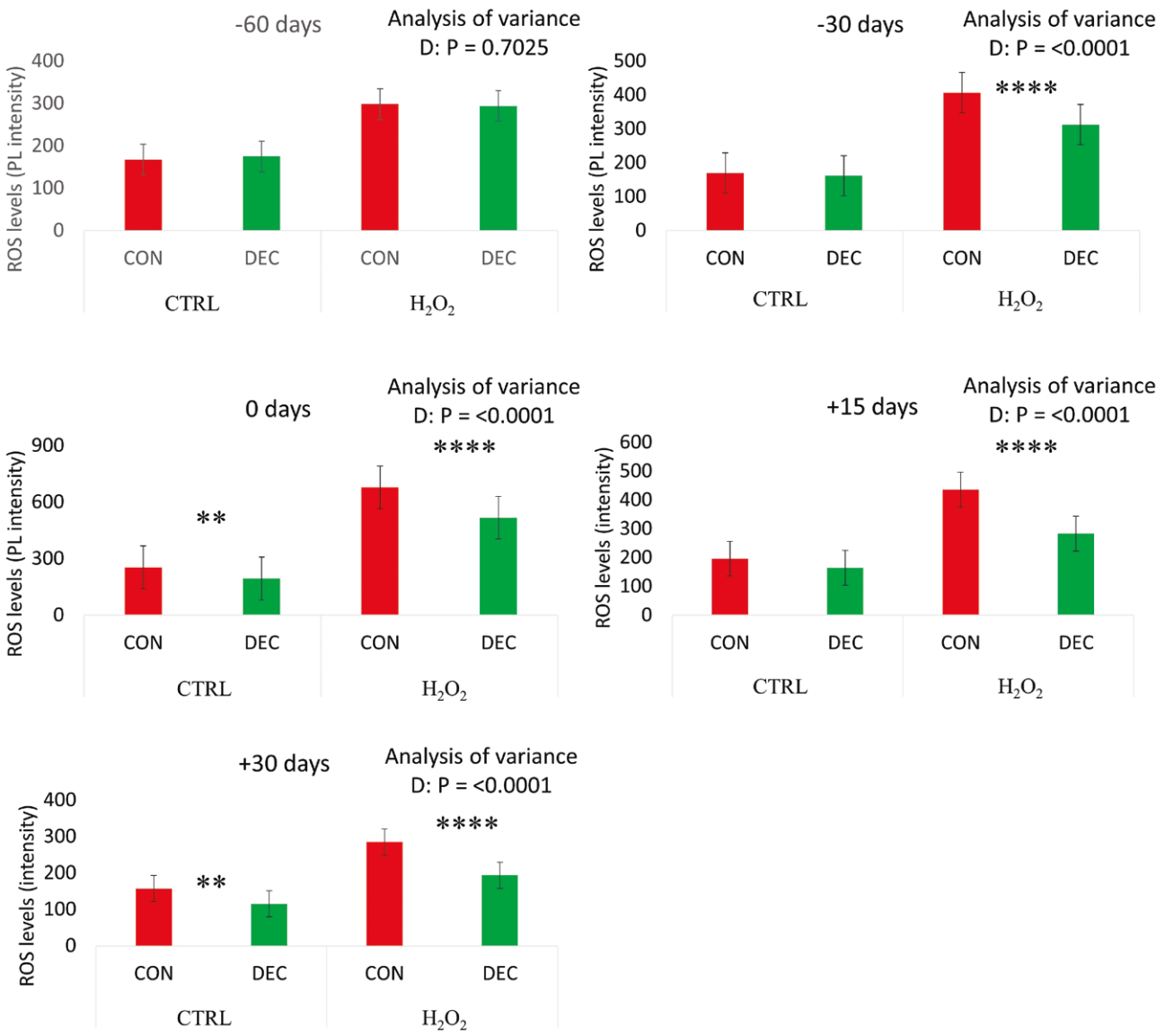

Figure 5. Production of reactive oxygen species (ROS) in peripheral blood mononuclear cells (PBMCs) from control group (CON) and Decosel group (DEC) during the trial. Histograms represent the ROS production in untreated (CTRL) and H_2O_2-treated PBMCs from the two groups of animals. Data are reported as LSM ± SEM. Differences between groups: $^{**}P < 0.01$, $^{****}P < 0.0001$. D, oral additive administration effect.

as vitamins C and E in their reduced (active) forms. These enzymes naturally tend to increase their activity during oxidative stress (Somagond et al., 2022) and act as an intracellular protection system able to regulate ROS accumulation in tissues (Noguer et al., 2012; Sordillo and Raphael, 2013). It is reported that SOD and GSPx levels decreased after calving both in dairy buffaloes (Singh et al., 2017) and dairy cows (Sharma et al., 2011). We observed an increasing trend of both SOD and GSPx activity up to calving in both groups. Then, consistently with other studies, a decreasing trend after calving in the CON group. Differently, Decosel administration induced higher SOD and GSPx plasma activity and they did not tend to decrease after calving as in the control buffaloes. So, additive administration seemed to lead to an enforcement of antioxidant defense mechanisms.

Lipid peroxidation is a nonenzymatic chain reaction based on the oxidation of mainly unsaturated fatty acids and is associated with the presence of ROS. It leads to the formation of lipid peroxides and other intermediates that may influence the properties of cell membranes and their physiological functions (Halliwell and Gutteridge, 1985). The increasing metabolic rate during the transition period requires more oxygen increasing the ROS production and lipid peroxidation, especially after calving, due to the increased metabolic activity for colostrum synthesis and the onset of lactation (Singh et al., 2017). TBARS are the most common intermediates of lipid oxidation processes. The lack of time effect on TBARS levels in our study during the transition period is certainly not supportive of increased lipid oxidation, similar to what has been reported in water buffalo by other authors (Sauerwein et al., 2020). However, FRAP levels showed a different trend between groups, with a lowering in the CON one after calving and, instead, stable levels in DEC group, enhancing the antioxidant capacity of these animals. In fact, it is interesting to note that the ROS levels in PBMC of DEC animals were lower compared with CON, in untreated PBMC as well as after stimulation with H_2O_2 during the transition period (from 30 d before calving to 30 DIM).

The effects of oxidation on the products are not very evident, and although there may be a higher level of enzymes in

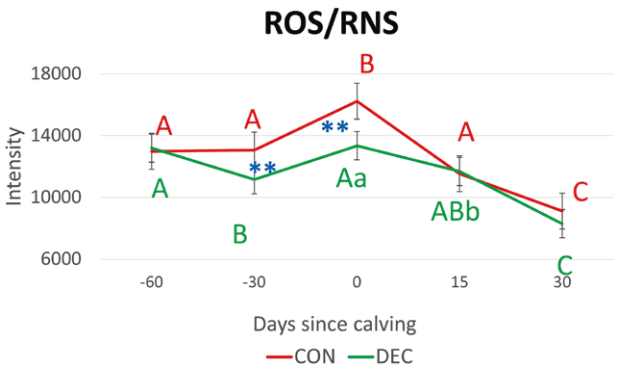

Figure 6. Total reactive oxygen species (ROS)/reactive nitrogen species (RNS) free radical activity in peripheral blood mononuclear cell (PBMCs) supernatants from control group (CON) and Decosel group (DEC) during treatment period. The ROS/RNS production expressed as photoluminescence (PL) intensity, in supernatants of PBMCs from the TWO groups of animals, collected after 20 h of incubation at 37 °C, 5% CO_2. Letters of same colors on timeline showed significant differences between times in the same group: $^{A,B,C}P < 0.01$; $^{a,b}P < 0.05$. Significant differences between groups are indicated with **($P < 0.01$). D, oral additive administration effect.

the plasma, this does not necessarily translate into a significantly greater enzymatic activity, but rather might represent a higher potential. This seems to be supported by the results of ROS levels in PBMCs.

Yeast can significantly reduce ROS synthesis in polymorphonuclear cells, enhancing a positive response in animals that experience inflammation status to cope with the oxidative stress (Jensen et al., 2008). Similarly, dietary supplementation of Decosel had positive effects on inflammatory signaling cascades. It is well known that an increase in ROS and RNS, combined with poor antioxidant defense, may result in cytotoxic events and oxidative stress-mediated macromolecular damage (Dinardo et al., 2022). Excessive exposure to ROS can cause severe oxidative damage to biological molecules including nucleic acids, proteins, and lipids (Lykkesfeldt and Svendsen, 2007; Ciampi et al., 2020). Our findings suggest that the reduction in ROS after H_2O_2 treatment reflects the antioxidant ability of the studied additive. In fact, the H_2O_2 stimulation is able to compromise the intrinsic antioxidant potential of cells by blunting catalase activity and leading to ROS accumulation (Akhter et al., 2019), thereby generating oxidative stress in cells (Asiaei et al., 2016). The supplementation of DE and yeasts appeared to reduce this negative effect, enhancing a proliferative effect on stimulated PBMC. In this regard, supplementation with Decosel exerts a proliferative effect on untreated and stimulated PBMCs (both with H_2O_2 and ConA) at calving, and this difference can be observed after ConA stimulation also during the early lactation (from 30 to 60 DIM). Therefore, there is a proliferative effect on PBMCs as well as an increase in their efficiency, aiding them to reduce ROS concentrations in case of activation. Moreover, the activation of the immune response requires an effective antioxidant system, because the higher the immune activation, the higher the production of ROS that needs to be metabolized by antioxidant systems (Hussain et al., 2016). These aspects could be considered of crucial importance for those diseases in which the inflammatory response exacerbates the deleterious effects of the illness. The balance between oxidative damage and antioxidant capacity is important for maintaining cell homeostasis and physiological activities (Domingues et al., 2016). Calving, and so the immediate transition moment, represents a stressing moment in which cells produce a large amount of ROS in response to the environmental change, leading to oxidative stress (Mutinati et al., 2014; Jang and Kim, 2019). The transition period affects energy metabolism, increases the production of ROS and RNS in dairy cattle (Sordillo and Raphael, 2013). Physiologically, animals have sufficient endogenous antioxidants to counteract the production of ROS/RNS which also plays a key role in gene activation, cell growth and death, or in the synthesis of bioactive substances such as prostaglandins (Abuelo et al., 2019). However, excessive increase in ROS/RNS may be due to the pathological conditions and/or the increase in physiological processes typical of the peripartum. When antioxidant system of cell, essentially represented by SOD, catalase, and GSPx, fails to maintain the balance between synthesized and catabolized ROS/RNS, a state of oxidative stress is established with consequent damage to cells or tissues or their functions (Dalle-Donne et al., 2005). The increased activity of endogenous plasma enzymes exerted by additive administration could justify the reduction of ROS/RSN accumulation especially with calving, the period most sensitive to oxidation.

The acute phase proteins are blood proteins that can be used to assess the innate immune system's systemic response to infection, inflammation, or trauma (Cerón et al., 2005). Not even inflammatory markers reveal differences during the peripartum period between experimental groups. The IL-1α, IL-6, and IL-8 and TNFα remained stable during the transition period in buffaloes but declined after the first 30 d of lactation, reporting values slightly lower than what was reported in the literature for buffaloes (Gomaa et al., 2021) and cows (Ishikawa et al., 2004). An increase of IL-6 and TNFα during early lactation has been detected by other authors, considering it a physiological adaptation to lactation (Bradford and Swartz, 2020), although a delay in resolution of this increase or signals can lead to a negative impact on the milk production, health, and fertility (Manimaran et al., 2016). In dairy cows, Hp has been used as a marker for various diseases including typical production diseases such as mastitis and fatty liver syndrome (Eckersall and Bell, 2010). The changes in concentration we observed herein for Hp are similar to those reported in dairy cows in which some authors observed a peak around calving, 10- to 20-fold of basal (Hachenberg et al., 2007; Saremi et al., 2012), but of lower intensity. Many authors reported similar results in buffaloes, with a peak of lower intensity than dairy cows at calving and then a decreasing trend (Deng et al., 2015; Sauerwein et al., 2020). Differently, in buffaloes, Gianesella et al. (2019) reported no difference between d −7 and +7, but still lower values at d +30 and +50 compared to times nearest to calving. However, Decosel addition reduces time between Hp peak and its lowering after calving. These results, although in the range reported by other authors for healthy buffaloes during the transition period (Horadagoda et al., 2001; Sauerwein et al., 2020), showed that the peripartal inflammatory status less intense compared to dairy cows and that Decosel addition is able to resolve it earlier after calving.

Conclusion

Based on the obtained results, we can conclude firstly that the transition period of buffaloes is not particularly stressful, evidenced by the virtual absence of production diseases

in early lactation, possibly due to a low metabolic challenge. Few parameters showed a real change during the transition period in buffaloes, in particular some antioxidant enzymes, PBMC viability, PBMC ROS production as well as Hp levels. For these patterns, in which probably the transition period can exert its maximum effect, there is the evidence of effectiveness of the commercial additive tested. Indeed, its dietary supplementation was able to enhance antioxidant enzyme activity, improve PBMC viability also after exogenous stimulation, and demonstrate an ability to resolve the inflammatory status typical of the peripartum period earlier.

Nevertheless, our results do not fully elucidate the underlying mechanism of the reported positive effects. It is possible that the timing of evaluation in our study was relatively extended, and there might be a need for further investigation with tighter timings to capture more subtle changes. Additionally, information about buffaloes' metabolic status during the transition period is still limited. While this study contributes to the understanding of the immune regulatory mechanisms of the commercial additive, more in-depth studies are required for a comprehensive mechanistic understanding of such supplementation.

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Author contributions

Aristide Maggiolino: Writing—original draft, Formal analysis, Data curation, Methodology, Resources. Maria Federica Sgarro: Methodology, Formal analysis, Data curation. Elisabetta Casalino: Writing—original draft, Methodology, Formal analysis, Supervision. Tiziana Latronico: Formal analysis, Data curation, Methodology, Writing—review & editing. Grazia Maria Liuzzi: Formal analysis, Data curation, Methodology. Pasquale De Palo: Conceptualization, Methodology, Resources, Writing—review & editing, Funding acquisition, Supervision.

Conflict of interest statement. The authors declare no real or perceived conflicts of interest.

Ethics approval

The trial was authorized by the Ethical Committee for Animal Welfare of Animals employed in scientific research of the Department of Veterinary Medicine of the University of Bari (approval no. 07/2020).

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

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