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RESEARCH ARTICLE

Impact of a plant feed additive containing vitamin C on Holstein cow's performance under heat stress conditions

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ABSTRACT

A phytobiotic with secondary metabolites and with vitamin C, elaborated with *Emblica officinalis* and *Ocimum sanctum* (polyherbal vitamin C), was evaluated for milk production, reproductive performance, cytokines, and reactive oxygen species (ROS) production in first calving Holstein cows under heat stress conditions. Two hundred primiparous cows with 20 ± 6 days in milk (initial body weight 521 ± 70.0 kg) were fed a basal diet (19.40% CP; 1.43 Mcal/kg Nel dry matter (DM)) and randomly assigned to one of the treatments: 0 or 20 g/d of the polyherbal vitamin C was integrated daily into the diet with the fresh total mixed ration directly in the feeder. The experiment lasted 131 days and measurements of production were made daily and milk composition every 30 days. Pregnancy was recorded at the first service and accumulated in the experimental period. Results showed that the production and composition of milk were not affected by the supplementation with polyherbal vitamin C, however, the accumulated gestation rate improved from 45.74 to 59.34% ($P = 0.06$), particularly that of the first service (20.21 to 34.06%; $P = 0.03$). Mastitis problems were reduced from 21.28 to 12.09% ($P = 0.09$). polyherbal vitamin C did not modify the production of IL-1β, while the serum levels of IL-6 decreased significantly ($P < 0.001$) compared to the unsupplemtented cows. All these results indicate that the polyherbal vitamin C can improve the gestation rate of Holstein dairy cattle and have beneficial health effects by reducing mastitis problems. Also, polyherbal vitamin C can exert an anti-inflammatory effect by lowering IL-6 levels and reducing ROS production.

Keywords: Feed plant additive; Phytobiotic; Vitamin C; Dairy cow

INTRODUCTION

Vitamin C is an important antioxidant and pro-oxidant, which is required for the synthesis of various hormones and neurotransmitters and is found in high concentrations in the brain and adrenal glands. It is a required cofactor in several enzymatic reactions, including the synthesis of carnitine, cholesterol, catecholamines, and collagen, and its participation in various components of the immune system has been recognized (Ranjan et al., 2012; Matsui, 2012; Akbari et al., 2016). Mammals have developed the ability to synthesize ascorbic acid in the liver and under normal conditions the requirement for vitamin C is met endogenously, however there are conditions where it can be limiting such as heat stress where blood levels of vitamin C are reduced in lactating cows (Padilla et al., 2006), in cows with mastitis (Weiss et al., 2004; Kleczkowski et al., 2005; Ranjan et al., 2005a), or with liver failure (Matsui, 2012). So, use of exogenous supplementation has been suggested (Ranjan et al., 2012).

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There are several ruminally protected vitamin C products for inclusion in the diet (Matsui, 2012) and there are evaluations in dairy cattle (Weiss, 2001; Padilla et al., 2006; Guo et al., 2017) that show potential benefits that may be greater in lactating cows under heat stress (Matsui, 2012). However, dairy farmers do not supplement with this nutrient presumably because few sources are available, they are expensive, and the benefits are not reflected in milk production. That is why the impacts of this vitamin must be evaluated in terms of its impact on health and fertility in longer periods (Gutiérrez et al., 2019).

Synthetic vitamin C products have stability problems. So, natural plant alternatives with nutraceutical properties have been evaluated to replace synthetic vitamins (Martínez-Aispuro et al., 2019; Mendoza et al., 2019; Gutiérrez et al., 2019) and those sources could be profitable. There are reports that a phytogenic containing *Phyllanthus emblica* (*Emblica officinalis*) has the same potential as vitamin C to reduce heat stress in buffalo and dairy cattle (Haq et al., 2013; Lakhani et al., 2017). Lozano et al. (2019) incorporated a polyherbal (C-Powder®) into the diet of finishing sheep, improving the carcass yield and the antioxidant capacity of the meat (Lozano-Sánchez et al., 2021).

The objective of this experiment was to evaluate the addition of a polyherbal mixture based on *Emblica officinalis* and *Ocimum sanctum* (*Ocimum tenuiflorum*) (polyherbal vitamin C) in the diet of first calving Holstein dairy cows in early lactation. Our hypothesis is that polyherbal vitamin C could improve milk production, reproductive performance, and reduce the veterinary problems in primiparous lactating cows under heat stress conditions.

MATERIALS AND METHODS

Cow's performance

The experiment was conducted in a dairy production unit located in the Mexican region known as "Comarca Lagunera" in the state of Durango (25° 39' 14.5 "N 103° 27' 28.4" W; altitude 1,122 meters) with an arid climate warm (BWh) and an average annual temperature higher than 18 °C (21.1 °C) under heat stress according to the temperature-humidity index (THI: 0.81 × Air temperature + ((relative air humidity/100) × (Air temperature - 14.4)) + 46.4)) (Ruíz-García et al., 2018), considering the critical value when the THI value exceeds 65. The THI values during the months that the experiment lasted (August to November 2018) by hour were in average: 64.43 at 7:00 h; 75.37 at 15:00 h; and 73.10 at 20:00 h; and only was below 65 during earling hours in October and November, which maintened the cows under heat strees.

Table 1: Composition of the basal diet of lactating Holstein cows in the dairy unit

Ingredients	%
Rolled corn	29.34
Cottonsed meal	13.28
Alfalfa	12.51
Corn silage	11.40
Barley distilled grains	9.38
Soybean meal	6.22
Alfalfa silage	5.98
Soybean hulls	5.27
Bypass protein (46.4% Crude Protein)	3.74
Mineral premix	1.59
Inert fat	1.26
Micotoxin adsorbent	0.04
Chemical composition	
Dry matter, %	51.64
Crude protein, %	19.6
Rumen degradable protein, %[a]	10.4
Neutral Detergent Fiber, %	43.7
Acid Detergent Fiber, %	26.5
NEl, Mcal/kg[a]	1.43

[a]NE, net energy, estimated with tables (NRC 2001).

Two hundred primiparous Holstein-Friesian cows with 20 ± 6 days in milk (initial live weight 521 ± 70.0 kg) were fed a basal diet (19.40% CP; 1.43 Mcal/kg NEl; NRC, 2001, Table 1) and 100 cows were assigned to the randomly to the following treatments: 0 or 20 g/d C-Powder® (Nuproxa Mexico, Nuproxa Switzerland, Indian Herbs Research & Supply Co. Ltd.). The polyherbal evaluated has vitamin C activity from gallic acid (5.1%), small hidrolizable tannins (12%) and asorbic acid (0.96%) (Lozano-Sanchez et al., 2021). Samples of the Polyherbal vitamin C were used to characterize the secondary metabolites by gas chromatography coupled to mass spectrometry (Table 2 and Fig. 1) as described by Roque-Jiménez et al (2020). In the feeder, the polyherbal vitamin C was mixed daily with the fresh total mixed ration. The experiment lasted 131 days and ended when the cows were 150 days in milk (DIM). Milk production was recorded daily using the automatic MDS-Milk® equipment and milk conductivity was checked daily in individual cows to diagnose of mastitis (Mlsna Dairy Supply, Inc.) (Rutten et al., 2013; Antanaitis et al., 2015) and on days 0, 24, 53, 88, and 116 of the experiment milk components (fat, protein, lactose, urea, and solid non-fat (SNF)) were analyzed with a Milkoscan. Individual veterinary treatments, diagnoses and number hospital visits were recorded in the database from day 20 to 150 DIM. This information was used to quantify the number of antibiotic doses, immunostimulants and vitamins received by each cow and the prevalence of diseases. Mastitis status was defined as a cow diagnosed and treated by the veterinarians in the farm.

Table 2: Chemical composition of the polyherbal C Powder® analyzed by GC-MS

N°	Compounds	tR[1]
1	N-Ethyl-2-phenethylamine	4.054
2	Cyclotrisiloxane, hexamethyl-	4.413
3	Oxirane, 3-ethyl-2,2-dimethyl-	4.630
4	Thymol	8.604
5	2-Propenoic acid, 2-methyl-, 1,2-thanediyil ester	8.733
6	Phenol, 2-methoxy-3-(2-propenyl)-	9.111
7	Diphenyl ether	9.478
8	Cycloisolongifolene, 8,9-dehydro-	9.685
9	2H-2,4a-Methanonaphthalene, 1,3,4,5,6,7-hexahydro-1,1,5,5-tetramethy	10.059
10	1H-Benzocycloheptene, 2,4a, 5,6,7,8	10.339
11	1,3-Cyclopentadiene, 1,2,3,4-tetra	10.373
12	2-Acetyl-6-methoxynaphthalene	10.432
13	Docosane	10.485
14	1-(6-Methoxynaphthalen-2-yl) ethanone	10.596
15	Aromadendrene, dehydro-	10.669
16	1,5,9-Cyclododecatriene, 1,5,9-trimethyl	10.809
17	3,4-Dimethylbenzyl isothiocyanate	11.149
18	Caryophyllene oxide	11.204
19	1,1,1-Trifluoro-2-(4-methylphenyl) propan-2-ol	11.347
20	2,4-Hexadiene, 2,3-dimethyl-	11.525
21	Cyclohexanespiro-5'-(2',4',4'-trimethyl-2'-oxazoline)	12.015
22	3-Penten-2-one, 4-bromo-	12.558
23	Quinoline, 1,2,3,4-tetrahydro-2-methyl-	12.811
24	2-Isopropylamino-4-methylbenzonitrile	12.981
25	1-Imidazol-1-yl-3-methylbut-2-en-1-one	13.735
26	1-Methoxy-3-(2-hydroxyethyl) nonane	14.774
27	n-Hexadecanoic acid	17.865
28	9,12-Octadecadienoic acid (Z, Z)-	24.062
29	9-Octadecenoic acid, (E)-	24.333
30	Hexadecanoic acid, 1,1-dimethylethyl ester	26.437
31	Hexatriacontane	31.130
32	Octadecanoic acid, butyl ester	32.954
33	Tetracosane	33.121
34	Indole-3-carbinol	34.023
35	Nonadecane	34.213
36	3-Indolethanamine	34.357
37	Bis (2-ethylhexyl) phthalate	34.634
38	Hexacosane	34.999
39	2-Ethylacridine	35.136
40	trans-2-Ethoxy-b-methyl-b-nitrostyrene	35.359
41	Propiophenone	35.475
42	Indolizine	35.530
43	Heptacosane	35.626
44	Octasiloxane	35.719
45	Docosane	36.223
46	2-Ethylacridine	36.325
47	Cyclotrisiloxane	36.442
48	Eicosane	36.854
49	Triacontane	37.539
50	Cyclotrisiloxane, hexamethyl-	38.010
51	Gamma-Tocopherol	38.101
52	Vitamin E	38.814
53	1,30-Triacontanediol	39.712
54	Heneicosane, 11-decyl-	40.273

(Contd...)

Table 2: ***(Continued)***

N°	Compounds	tR[1]
55	Gamma-Sitosterol	40.950
56	1H-Indole, 2-methyl-3-phenyl-	41.030
57	Nonacosane	41.534
58	Taraxasterol	42.115
59	Hexatriacontane	43.053

[1]Retention time.

Cytokines analysis, macrophage culture and reactive oxygen species (ROS) production

Blood samples were collected on day 100 after parturition from healthy cows for cytokines analysis and macrophage culture from peripheral blood mononuclear cells (PBMCs) isolation. The levels of plasma interleukin-1beta (IL-1β) and interleukin-6 (IL-6) were evaluated in samples from 11 cows per treatment. The concentrations of IL-1β and IL-6 were analyzed using commercially available ELISA kits specific for the bovine species (Pierce, Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instruction. All the samples were analyzed in duplicate.

Macrophages were obtained from PBMCs, as described by Benítez-Guzmán et al. (2019). Blood was collected from the caudal vein into 20 mL vacuum tubes with acid-citrate-dextrose solution and maintained at 4 °C, blood samples were transferred into 50 mL conical tubes and centrifuged at 10000×g for 30 min at room temperature. Buffy coats were diluted in 30 mL of citrated PBS pH 7.4, layered onto 15 mL of Percoll (Pharmacia, Uppsala, Sweden) at a specific density of 1.077, and centrifuged at 1200×g for 25 min. PBMCs were then removed from the interface between the plasma and Percoll solution at specific density of 1.077, pooled, diluted in 50 mL of citrated phosphate buffered saline (PBS) (130 mM trisodium citric acid, 5% BSA and PBS0), and centrifuged at 500×g for 15 min. The cell pellets were then washed three times with citrated PBS at 500×g for 10 min, suspended in Roswell Park Memorial Institute media (RPMI) (Gibco, New York, NY, USA) supplemented with 5 mM L-glutamine (Gibco, New York, NY, USA), 5 mM non-essential amino acids, 5 mM sodium pyruvate (Gibco, New York, NY, USA) and 20 mM sodium bicarbonate (CRPMI) containing 4% autologous serum to facilitate the adherence, and cultured overnight at 37°C and 5% CO_2. Non-adherent cells were then removed by three washes with prewarmed PBS, and adherent monocytes were cultured, as described previously, in CRPMI plus 12.5% autologous serum for 12 days until they differentiated to macrophages. Flasks were chilled on ice for 45 min and macrophages were harvested by repeated gentle pipetting and suspended at densities indicated on each experiment.

Reactive Oxygen Species (ROS) production was determined via the nitro blue tetrazolium (NBT) reduction as described previously (Martinez-Cortés et al., 2018). Macrophages were plated in 24-well plates. Macrophage priming was achieved by culturing cells with 100 ng/mL of Lipopolysaccharide (LPS) from Salmonella L6511 (Sigma Chemical Company, St. Louis, MO) in CRPMI plus 12% of autologous serum for 22 h previous to analyze of ROS. Subsequently, 20 μL of NBT (Sigma Aldrich) (1 mg/mL) were added to every well and incubated for 20 min in 5% CO_2 at 37 °C. Finally, a mix of 54 μL of 2 M KOH and 46 μL of dimethyl sulfoxide (Sigma-Aldrich) were added to dissolve formazan crystals and the formazan solution was transferred to a 96-well plate. The optical density (OD) was measured with a microplate spectrophotometer at 620 nm (multiskan GO plate reader, Thermo Fisher Scientific).

Statistical analyses

The normal distribution was tested in response variables with the Shapiro-Wilk test. Baseline body weight was used as a covariate for daily weight gain, final weight and milk yield (Steel et al., 1997). Milk yield and composition were analyzed with a linear mixed model. Initial body weight was not significant, therefore, the model with repeated measures included treatment effect (fixed), time of lactation (fixed), and cows (random effect) within treatment (Littell et al., 1998). Biochemical variables measured once included treatment and random error in the model. Pregnancy rates and disease incidences were analyzed with the Chi-square test with Software R (Shim et al., 2019).

RESULTS

Dairy cattle performance

Milk production and composition were not affected by the dietary supplementation with the polyherbal vitamin C (P> 0.05; Table 3). Even when initial body weight had a significant effect as a covariate on body weight and daily live weight changes, the polyherbal had no effect on those variables (Table 3), while the pregnancy rate was improved particularly from the first service, improving from 20.21 to 34.06% (P = 0.03) and in the accumulated increasing from 45.74 to 59.34% (P = 0.06). Regarding the effects of polyherbal vitamin C on animal health, no changes were found in body weight, abortions or mortality, but a considerable reduction of mastitis incidence was observed from 21.28 to 12.09% (P = 0.09) (Table 4). These results indicate that the polyherbal vitamin C reduces mastitis problems and improves reproductive parameters in lactating cows subjected to heat stress.

Anti-inflammatory effect of polyherbal vitamin C

The inflammatory cytokines IL-1β and IL-6 (Fig. 2) were detected in the serum of both groups of cows. Polyherbal vitamin C did not modify the production of IL-1β (Fig. 2A), while the levels of IL-6 decreased significantly (P <0.001, Fig. 2B) compared to the control group. Table 5 shows the mean and differences of both groups.

The polyherbal vitamin C did not modify the macrophage basal ROS levels compared to those of the control group (Fig. 3). It was also observed a significant production of ROS in macrophages from the control group stimulated with LPS. Interestingly, when macrophages stimulated from cows that consumed the polyherbal vitamin C, it was detected a decrease in ROS production compared to those from the control group, indicating that the polyherbal vitamin C can exert an anti-inflammatory effect by lowering IL-6 levels and reducing ROS production (Table 6).

DISCUSSION

Dairy cattle performance

Previous studies have shown that lactating cows subjected to heat stress considerably decrease plasma concentrations

Table 3: Least square means for milk production and composition in primiparous Holstein cows with or without polyherbal vitamin C supplementation

	Control	C-Powder® 20 g/cow/d	SEM	*P*-value for effect of treatment
n	94	91		
Milk production, kg/d	35.60	35.32	0.594	0.73
Milk composition, g/kg				
Fat	32.78	33.94	1.684	0.63
Protein	30.62	30.58	0.587	0.96
Solid non fat	89.46	89.22	0.585	0.78
Lactose	50.50	50.98	0.716	0.64
Urea, mg/dl	109.70	115.40	7.441	0.60
Initial BW kg, at 20 DIM	576.81	570.39	3.024	0.30
BW kg, at 66 DIM	491.92	499.21	2.942	0.22
BW kg, at 90 DIM	488.59	490.18	2.936	0.79
BW kg, at 120 DIM	492.55	491.56	2.982	0.62
BW kg, at 150 DIM	505.63	507.18	2.849	0.78
Live weight changes, kg/d	-0.543	-0.482	0.029	0.14

SEM, standard error of the mean; DIM, days in milk.

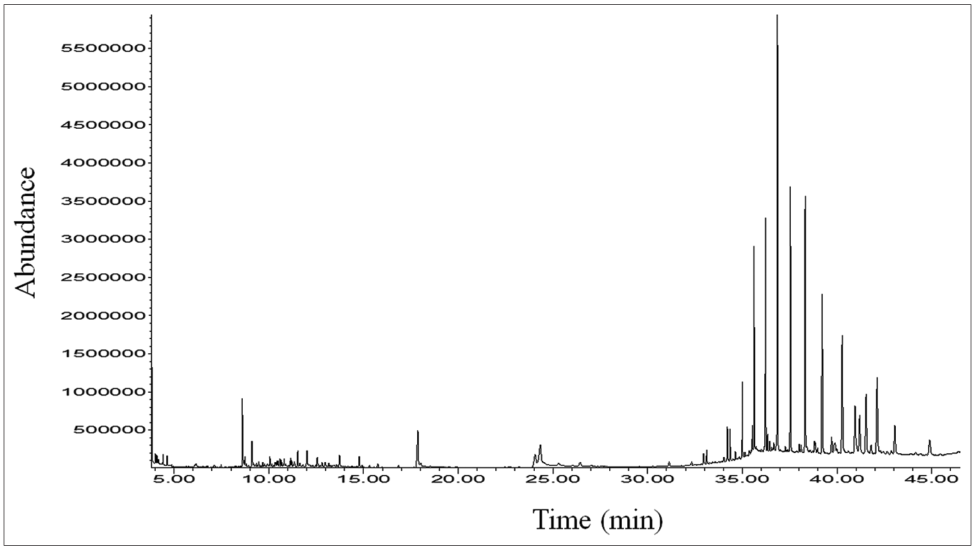

Fig 1. Total ion chromatogram of the volatile components and chemical composition in the Polyherbal vitamin C. Chemical composition of C Powder® by CG-MS

Table 4: Effect of Polyherbal vitamin C supplementation on reproductive parameters and health events (%) of Holstein cows

Parameter	Control	C-Powder® 20 g/cow/d	Additive difference %	Chi-squared	*P*-value for effect of treatment
Gestation rate, %					
1st service	20.87	59.34	+38.47	28.408	0.0001
Accumulated	45.74	59.34	+13.60	3.410	0.06
Diseases prevalence, %					
Abortions	4.25	6.59	+2.34	0.49	0.48
Lameness	7.45	10.49	+3.54	0.69	0.40
Mastitis	21.28	12.09	-9.19	2.784	0.09
Respiratory	23.40	16.48	-6.92	1.377	0.24
Fever (non specific)	4.26	4.40	+0.14	0.002	0.96
Mortality	5.26	8.69	+3.0	0.84	0.35

Table 5: Least square means for interleukins IL-6 and IL-1beta (IL-1β) in bovine serum from in primiparous Holstein cows with or without polyherbal vitamin C supplementation

	Control	C-Powder® 20 g/cow/d	SEM	P-value for effect of treatment
IL-1β (pg/ml)	48.35	48.40	2.752	0.98
IL-6 (pg/ml)	79.22	78.21	0.169	0.0004

SEM, standard error of the mean.

Table 6: Least square means for ROS production in bovine macrophages stimulated with LPS from primiparous Holstein cows with or without polyherbal vitamin C supplementation

	Control	C-Powder® 20 g/cow/d	SEM	*P*-value
Cells without LPS (OD)	0.1350	0.1412	0.0405	0.98
Cells with LPS (OD)	0.3284	0.1900	0.0326	0.001

LPS, Lipopolysaccharide; OD, optical density; SEM, standard error of the mean.

of vitamin C (Miller and Brezezinska-Slebodzinska, 1993; Padilla et al., 2006), however, as observed in this experiment, the impact of supplementing with polyherbal vitamin C did not improve milk production. Some studies report a positive correlation between the performance of milk components (protein, lactose and SNF) with the concentration of ascorbic acid in plasma (Tanaka et al., 2008), but others report that there is no correlation (Santos et al., 2001). The exposure of cows to heat stress during the experiment could be a limitation for the cows to show changes in milk production, which is aggravated because high-performance cows must dissipate the metabolic heat associated with intake, fermentation heat (Mendoza et al., 2003) and caloric increment of the metabolism related to the synthesis and secretion of milk (Wolfenson and Roth, 2019).

Oxidative stress increases in dairy cattle after calving and under conditions of heat stress and total ascorbic acid in plasma decreases significantly during the peripartum period (Tanaka et al., 2011). It has been suggested that under conditions of heat stress, the availability of vitamin C in dairy cows is important to successfully adapt to stress as suggested by experimental data with goats (Sivakumar et al., 2010) and cattle (Ranjan et al., 2012).

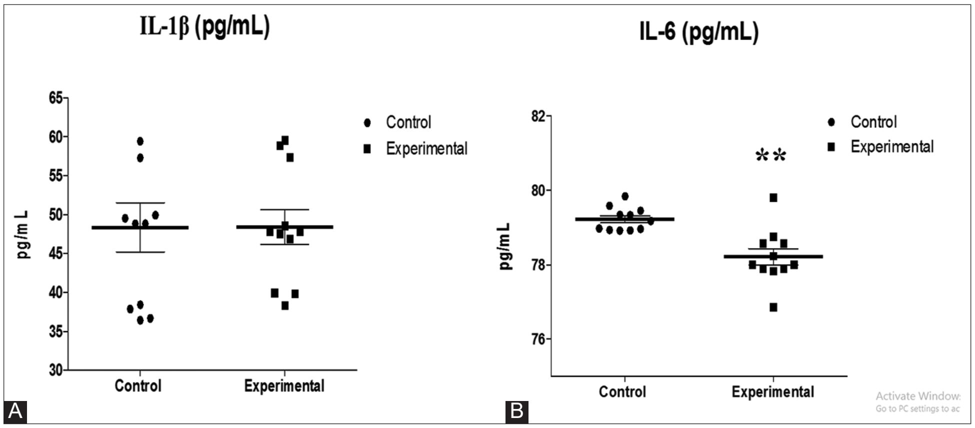

Fig 2. Effect of Polyherbal vitamin C on the bovine serum leves of the inflammatory cytokines IL-1β and IL-6. A) Serum IL-1β levels of cows that received (Experimental) or not (Control) the C Powder® supplement. B) Serum IL-6 levels of cows that received (Experimental) or not (Control) the C Powder® supplement.

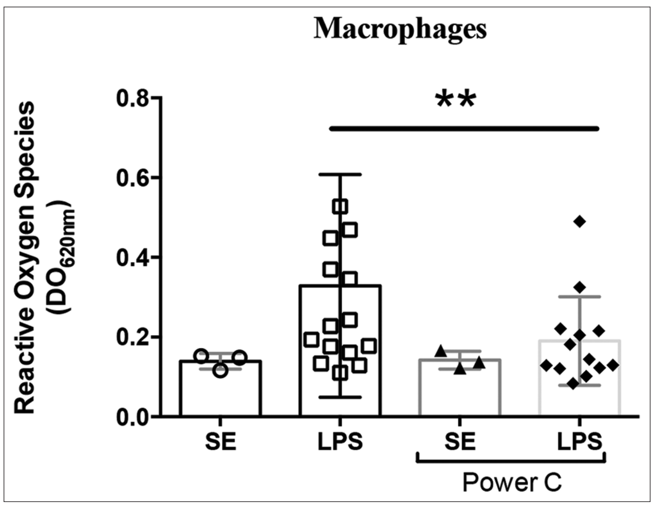

Fig 3. Polyherbal vitamin C decreases ROS production in bovine macrophages stimulated with LPS. A) The Reactive Oxygen Species (ROS) were detected in macrophages derived from monumental cells of peripheral blood of cows that received or not the C Powder® supplement. Some macrophages were stimulated with Lipopolysaccharide (LPS). SE, unstimulated macrophages; ***P* <0.001; OD, Optical Densities.

The positive responses observed in fertility seem not to be associated with the liveweight changes even when polyherbal vitamin C supplemented cows tended to lose less weight (-0.482 kg/d) than the control group (-0.543 kg/d). Experiments with beef cattle show no response in weight gain have to vitamin C however, some changes have been observed in body fat, suggesting that vitamin C may affect lipid metabolism (Oohashi et al., 2000; Mori et al., 2006; Pogge and Hansen, 2012; Pogge and Hansen, 2013; Matsuda and Takahashi, 2014). In the polyherbal plants (*Emblica officinalis* and *Ocimum sanctum*).

Polyherbal vitamin C plants contains secondary metabolites such as Thymol and Eugenol, and others (Khan, 2009; Joshi et al., 2011) that have metabolic activity that can influence cholesterol regulations (Ovesnaâ et al., 2004; Traber and Atkinson, 2007; Mastelić et al., 2008; Vance et al., 2013; Singh, 2013) which could have an impact in cholesterol metabolism in cows (Gross et al., 2015) and deserves further exploration. Thymol and Eugenol have also antioxidant properties (Mastelić et al., 2008) and antimicrobial activity (Rhayour et al., 2002; Michiels et al., 2007; Gutiérrez-Larraínzar et al., 2012).

There are reports where vitamin C has improved the pregnancy rate, the reproductive performance of cattle and fertility in humans (Ranjan et al., 2012). The different functions of vitamin C may explain the improvements in fertility observed in the supplemented cows with polyherbal. It can be speculated that the antioxidant effects help to give resistance to heat stress or that its additional availability had an effect at the hormonal level; vitamin C is required for the synthesis of a large group of hormones and neurotransmitters and plays a very important role in the biosynthesis of steroids (Luck and Zhao, 1993). Ranjan et al. (2012) reviewed the processes where vitamin C can influence neuroendocrine regulation, highlighting, norepinephrine biosynthesis; as a reducing agent for the synthesis of aldosterone in the adrenal gland, the release of corticosteroids in the bovine adrenal gland, and the regulation of oxytocin biosynthesis and release.

The higher fertility at the first service with the polyherbal mix reflects a better physiological condition in the conception rate. Vitamin C may be involved in some ovarian functions and integrity; it has been reported that there is a relationship between the size of the ovaries and their concentration of ascorbic acid (Khan and Das, 2012). An *in vitro* study showed that ascorbic acid is important for maintaining the integrity of the follicle and may be involved in regulating the regeneration of the extracellular matrix in cattle (Thomas et al., 2001). After conception, vitamin C may help maintain pregnancy. *In vitro* studies reported that ascorbic acid improved buffalo embryo development (Saikhun et al., 2008). In this study, pregnancy and delivery were not recorded, but beneficial effects could be expected in these periods; lower concentrations of ascorbic acid have been reported in retained placental tissues in animals that reported placental retention (Kankofer, 2001).

The reduction in mastitis with polyherbal with vitamin C activity is consistent with other reports. Dietary supplementation with ascorbyl-2-polyphosphate decreased the somatic cell count in the milk of cows with endotoxin-induced mastitis (Weiss and Hogan, 2007). Padilla et al. (2006) found that heat stress decreases the plasma concentration of vitamin C, as did mastitis in dairy cows (Weiss et al., 2004; Kleczkowski et al., 2005; Ranjan et al., 2005a). In other studies with cattle and goats, the lower content of ascorbic acid in plasma has been associated with the presence of mastitis (Steffert, 1993; Gupta et al., 1999; Ranjan et al., 2005a) and there is information where the administration of vitamin C in the mastitis treatment improves response in cattle (Chaiyotwittayakun et al., 2002; Naresh et al., 2002; Ranjan et al., 2005b). Weiss and Hogan (2007) supplemented vitamin C (30 g/d) and observed a reduction in the somatic cell count. There are also reports where there was no relationship between somatic counts and plasma vitamin C without supplementation (Santos et al., 2001) or where the intramammary response by vitamin C (25 g intravenous) was limited under conditions of exposure to endotoxins (Chaiyotwittayakun et al., 2002).

There were no effects of herbal C in the incidence of diseases as observed with other nutraceutical polyherbal products (Gutiérrez et al., 2019) and a greater impact on other diseases was expected due to reports por reports with vitamin C (Hemilä, 2017). Respiratory problems in cows did not show significant differences, but numerically they had a reduction of 4.69%. In humans, five controlled trials found significant effects of vitamin C against pneumonia (Hemilä, 2017). Most of the studies in dairy cattle have been carried out under heat stress conditions but polyherbal vitamin C could have benefits in cold climates considering that in humans and rodents vitamin C can protect against stress caused by hot and cold environments (Le Blanc et al., 1954; Dugal, 1961; Strydom et al., 1976; Chang et al., 2016).

Heat stress has been shown to have negative effects on the secretory function of the mammary gland (Silanikove et al., 2009) and to decrease the efficiency of energy use for productive purposes (Rhoads et al., 2009; Wheelock et al., 2010). In bovines under heat stress, available energy is the most limiting nutrient for lactating dairy cows (West, 2003). In response to elevated temperatures, *in vitro* epithelial cells of the bovine mammary gland show abnormal morphology and show a reduction in cell proliferation (Collier et al., 2006), suggesting that hyperthermia induced by heat stress compromises the function of mammary cells.

Although there is no vitamin C deficiency in dairy cattle, it is known that, in some conditions, animals may not necessarily synthesize sufficient amounts of ascorbic acid to reach optimal levels that prevent infection (Hemilä, 2017). Its deficiency reduces resistance against infections and the inflammatory response, and its supplementation improves the activity of antimicrobial and natural killer cells, improves the phagocytic capacity of neutrophils and protection against oxidative damage, supports the proliferation of lymphocytes and the production of interferon and contributes to the maintenance of the integrity of oxide reduction cells (Ranjan et al., 2012; Akbari et al., 2016).

In intensive dairy farms, it has been considered that the first problem that generates economic losses is mastitis (clinical-subclinical), followed by reproductive disorders (Ózsvári, 2017). The herbal containing vitamin C could help to reduce economic losses due to reduced yield and discarded milk that can represent twice the costs of drugs in mastitis (Seegers et al., 2003) and reduce veterinary costs for mastitis treatments and the fees of veterinary doctors (Ghule et al., 2012).

Polyherbal vitamin C supplementation improved fertility at the first service which could reduce the interval between calvings. The estimated losses due to increasing the calving interval in dairy cattle can be USD 3 per cow/day (Ózsvári, 2017) and the estimates by De Vries (2006) range from USD 3.2 to 5.1/cow/day in dairy farms in the United States and some models indicate that these values may be underestimated by not considering all losses (milk production, fetal losses, additional reproductive interventions and replacement costs of cows due to infertility), estimating that the losses can increase from USD 5.77 to 6.11/cow/day. Currently, ascorbic acid supplementation in dairy cattle diets in commercial stables is not common due to complications such as vitamin stability, availability of products protected from degradation and costs, but the polyherbal mixture seems to be a viable alternative for its inclusion in large scale quantities.

Cytokines analysis, macrophage culture and reactive oxygen species (ROS) production

The favorable impact that was found on the pregnancy rate and the reduction in the incidence of mastitis using polyherbal vitamin C could be due to the decrease in inflammatory parameters (IL-6 and ROS) in lactating cows subjected to heat stress. This is because dairy cows exposed to heat stress conditions are known to exhibit an imbalance in animal health, including dysregulation of the immune response (Bagath et al., 2019). This environmental condition promotes a pro-inflammatory state in cows. Further, the increase of a pro-inflammatory environment is related to the alteration of the metabolism and the excessive mobilization of fats, which are also initiated by the invasion of pathogens, tissue damage and excessive fat deposition (O'Boyle et al., 2006; Loor et al., 2013). It is widely known that during the postpartum period of cows a systemic inflammation occurs with alteration of metabolism, this is due to various factors such as excessive fat mobilization leading to the production of pro-inflammatory cytokines, tumor necrosis factor alpha (TNF)-α, and IL-6 (Trevisi et al., 2012; Bradford et al., 2015). Proinflammatory cytokines inhibit intracellular signaling of insulin, heightening insulin insensitivity and exacerbating the release of non-esterified fatty acids (NEFA) from adipose tissue (De Koster et al., 2018) also stimulate the production of acute phase proteins such as haptoglobin, which is commonly used as a biomarker of inflammation (Eckersall and Bell, 2010; Huzzey et al., 2011; McCarthy et al., 2016).

Elevated concentrations of TNF-α, IL-1β and IL-6 accompanied by insulin resistance resemble sterile inflammation and metabolic syndrome (Slattery et al., 2004; Fontana et al., 2007), The metabolic alteration found in dairy cattle is mainly due to the homeoretic change for milk production, when nutrient demand exceeds dietary intake, resulting in a state of negative energy balance (NEB) (Drackley, 1999). The presence of biomarkers of metabolic stress and inflammation in dairy cows has been associated with diseases of the reproductive and respiratory systems and mastitis (Osorio et al., 2014). The proinflammatory cytokines IL-6, IL-1, and TNF-α are commonly used to measure liver function in cows (Huzzey et al., 2011). Thus, IL-6 has been found to be elevated in periparturient cows with liver dysfunction and in early lactation cows with ketosis (Loor et al., 2007). Also, in metritis and endometritis, high concentrations of haptoglobin, IL-1β and IL-6 have been found in cow serum, before the disease can be diagnosed (Huzzey et al., 2011).

In this way, several studies have shown the anti-inflammatory effects of natural products that were supplemented in the diet of cows (Gessner et al., 2017) exposed to heat stress (Hashemzadeh et al., 2014). This study demonstrated that dietary supplementation with polyherbal vitamin C in cows exposed to heat stress significantly decreased serum IL-6 levels. Which can lead to greater control over animal health, with a lower incidence of mastitis and respiratory problems. Further, this result can explain the effect of the supplementation in health parameters, in general the animals show 12% of mastitis and 16% of respiratory problems less than control groups, this effect. These data suggest that polyherbal vitamin C has an important effect in the regulation of metabolic status of animals, Tocopherol (Vance et al., 2013), and Gamma-sitosterol (Singh, 2013), and Naproxen has an impact effect in the regulation of cyclooxygenases with narrow relation of IL-6 regulation (Duggan et al., 2010).

Heat stress is known to increase the production of ROS and presence of several antioxidants in the polyherbal vitamin C could have an effect on ROS production in macrophages derived from PBMCs. Metabolic stress in cows causes an alteration in physiological homeostasis (Abuelo et al., 2015), increasing the mobilization of body reserves from adipose tissue, generating a negative energy balance, immune and inflammatory dysfunction and increased production of ROS and species of reactive nitrogen (Abuelo et al., 2019).

The decrease in macrophage ROS production activated by LPS from cows supplemented by polyherbal vitamin C compared with macrophage from control, suggest that polyherbal vitamin C has an important effect in the regulation of ROS production and it could be important in the regulation of oxidative stress. This could be possible because the effect of all components of polyherbal vitamin C described before with antioxidant activity like vitamins A, E and vitamin C (Bernabucci et al., 2014). Therefore, the regulation of ROS production that was observed in this study when using polyherbal vitamin C under conditions of heat stress can be reflected in the physiological equilibrium shown by the experimental cows.

Several studies have determined the beneficial effect of vitamin C supplementation in the diet of lactating cows, showing the need to generate new products that maintain the stability of this vitamin (Hiatt et al., 2010; Matsui, 2012; Ranjan et al., 2012).

A major constituent of the polyherbal vitamin C is *Phyllanthus emblica* (also known as *Emblica officinalis*) known as Amla in Ayurvedic medicine, a plant reported to contains ascorbic acid (Khopde et al., 2001; Scartezzini et al., 2006) and significant levels of gallo-tannoids that have vitamin C activity (Pozharitskaya et al., 2007) with antioxidant activity (Velderrain-Rodríguez et al., 2018). The *Phyllanthus emblica* plant that contains Emblicanin A, Emblicanin B, Pumigluconin, Pedunculagin, Gallo-ellagitannins and Rutin, which in studies with rats have shown outstanding antioxidant activity (Sabu and Kuttan, 2002; Bhattacharya et al., 2002; Govindarajan et al., 2005) and the two emblicanins enhanced the efficacy of vitamin C in reducing dehydroascorbic acid to ascorbic acid (Ghosal et al., 1996; Bhattacharya et al., 2000; Scartezzini et al., 2006). The other plant *Ocimum tenuiflorum* is also a medicinal plant recognized by its essential oils contents, phenols and flavonoids (Shackelford et al., 2009). The metabolites in the polyherbal vitamin C plants contains natural antioxidants, metabolites with bactericidal, anti-inflammatory properties, and others that can help improve animal well-being or reduce stress under certain conditions (Humer et al., 2018).

CONCLUSIONS

The polyherbal vitamin C with vitamin C activity in doses of 20 g/d of in primiparous Holstein cows in early lactation under caloric stress conditions, improved gestation rate, and reduced mastitis problems. The metabolites with antioxidant activity in the polyherbal manifested an important effect in the regulation of ROS production and it could be important in the regulation of oxidative stress together with the regulation of proinflammatories citokynes in cows under heat stress. Polyherbal vitamin C supplementation providing compounds with vitamin C activity, natural antioxidants and other with antimicrobials properties could have an important activity like metabolic modulator and inflammatory regulator in animals under important fisiological stress like fresh cows.

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Authors' Contributions

All authors participated equally in the design of the experiment, analysis and writing of the manuscript.

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