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REGULAR ARTICLES

Addition of clinoptilolite in the diet reduces uterine PMN leukocytes and open days in multiparous lactating dairy cows managed in a mountain tropical pasture-based system

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Abstract

This study aimed to assess the effect of adding clinoptilolite in the diet on uterine health and reproductive performance in multiparous lactating dairy cows managed in a tropical pasture-based system above 2500 m of altitude. Seventy-seven multiparous Holstein crossbred cows from two farms were allocated randomly into two groups: clinoptilolite supplemented cows (CLG, $n=42$) and non-supplemented cows as control (CG, $n=35$). Cows from CLG were supplemented with clinoptilolite from 30 days (50 g/cow/day) before to 60 days after calving (200 g/cow/day). In CLG cows, percentages of uterine PMN leukocytes ($P<0.0001$) and proportion of subclinical endometritis ($P=0.0187$) were lower than in CG. The interval calving to first corpus luteum was shorter ($P=0.0759$) in CLG than CG, and calving to first service interval was similar between treatments. Cows from CLG became pregnant 35 days earlier than CG cows ($P=0.0224$). On farm A, calving to conception interval was 18.1 days longer in CLG than in CG ($P=0.3750$); in farm B, this interval was 86.2 days shorter in CLG than in CG ($P=0.0002$). In conclusion, daily addition of clinoptilolite in the diet decreased the percentage of uterine PMN leukocytes, the proportion of cows with subclinical endometritis, and shortened the calving-conception interval in multiparous lactating dairy cows.

Keywords Clinoptilolite · Dairy cows · Uterine PMN leukocytes · Reproductive performance · Tropic

Introduction

In recent decades, a dramatic decline in fertility rates has been observed worldwide in dairy cattle (Szenci et al. 2018). Hence, monitoring the peripartum period is essential to maintain and/or improve reproductive performance in dairy cattle. Proper management of dairy cows before and after calving, particularly in the first 100 days of milking, is essential to achieve optimal reproductive performance in the dairy herd (Szenci et al. 2018). The transition period extends from 6 to 8 weeks before the end of gestation to the onset of lactation (Roche et al. 2017). In this phase, dairy cattle shift from a physiological stage of gestation in which no milk is produced to one of non-pregnancy and intense milk production (Drackley 1999). During the transition, important hormonal and metabolic changes occur. Cows undergo a process of adaptation of the digestive system and high demand for nutrients due to fetal growth and milk synthesis (Zebeli et al. 2015). This physiological stage leads the cows to develop a state of oxidative stress and

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negative energy balance that significantly reduces voluntary dry matter intake and depresses the immune system (Sordillo, et al. 2009; Zebeli et al. 2015). This physiological, metabolic, and nutritional context favors occurrence of numerous disorders such as ketosis, placental retention, abomasal displacement, and bacterial infections such as mastitis and endometritis. These conditions seriously affect productive and reproductive performance (Mulligan and Doherty 2008) and cause death or culling of around 10% of affected cows (Pinedo et al. 2010).

Subclinical endometritis, an inflammatory disease of the reproductive tract, occurs very frequently in early postpartum in dairy cattle, because of the natural lacerations to superficial layer of the endometrium during calving and concomitant bacterial contamination of the uterus (Pascottini and LeBlanc 2020). This situation is aggravated because the immune system is depressed during the peripartum period (Sordillo et al 2009). Likewise, as this reproductive disorder occurs without clinical symptoms, it is not detected or treated, increasing the risk of developing more serious and difficult-to-treat uterine diseases (Bromfield et al. 2015). In addition, subclinical endometritis reduces reproductive efficiency (Quintela et al. 2018), increases the risk of cows being culled due to infertility (LeBlanc et al. 2002), and causes significant economic losses in the herd (Inchaisri et al. 2010).

Considerable attention has been paid to nutritional management of cows during transition (Drackley and Cardoso 2014; Lacasse et al. 2018). The addition of some supplements in diets to meet the nutritional needs of dairy cows in transition (Little et al. 2017) has contributed to reducing adverse effects during this critical period and has favored milk production, reproduction, and herd health (Osorio et al. 2016; Little et al. 2017).

Zeolites are natural minerals of volcanic origin described as crystalline hydrated aluminosilicates of alkali and alkaline earth cations having an infinite and open three-dimensional structure (Ivkovic et al. 2004). These chemical and physical characteristics confer catalytic, adsorption, and ion exchange properties to zeolites (Valpotić et al. 2017). Among natural zeolites, clinoptilolite has been used as a feed supplement in zootechnical and biomedical fields with multiple beneficial effects (Valpotić et al. 2017, 2018; Folnožić et al. 2019). Addition of zeolite to a cattle diet modified the molar ratio of volatile fatty acids, decreasing acetate and increasing propionate, which may have a positive effect on energy efficiency (Urías-Estrada et al. 2017). Daily administration of 200 g of clinoptilolite to heifers from day 210 of gestation until the end of first lactation significantly increased body condition, serum concentration of glucose, milk production, and reproductive variables (Karatzia et al. 2013). In a previous study, a greater proportion of multiparous Holstein cows supplemented with zeolite in pre- and post-partum (180 and 270 g, respectively) achieved better body condition at 45 days postpartum, a follicle ≥ 10 mm was detected at 35 days postpartum and had a lower percentage of PMN leukocytes in the uterus compared to non-supplemented group (Garzón et al. 2017).

The stimulating effect of clinoptilolite on the immune system has been also documented in several studies in cattle. For instance, in Holstein cows, addition of 3% clinoptilolite to the basal diet, from 4 weeks prior to expected parturition until 12 weeks after calving, increased milk production and decreased somatic cell count (Ural 2014). In a recent study, supplementation of Holstein cows with 100 g of clinoptilolite daily from 7 months of pregnancy to day 75 postpartum (Đuričić et al. 2020a), decreased the number of bacteria species causing mastitis and the number of affected quarters, as well the risk of intramammary infection compared to control group (Đuričić et al. 2017, 2020b).

Information is scarce about the effect of zeolites on reproductive activity in cattle. This study aimed to evaluate the effect of adding clinoptilolite in the diet, from 30 days before to 60 days after calving, on uterine health and reproductive performance in multiparous lactating dairy cows managed in a pasture-based system above 2500 m of altitude, in the southern Ecuadorian Andean highlands.

Materials and methods

Study location

This study was conducted in two commercial dairy farms located at 15 to 20 km from the city of Cuenca, Azuay Province, Ecuador, over 2500 m above sea level (masl). The area has an average temperature of 12.3 °C and a rainfall of 1080 mm/year. These dairy farms had, in general, a similar management system, based on grazing, mechanical milking (twice daily), and a milk production of approximately 5000 kg/year. Farm A is located at 2680 masl and farm B at 3158 masl.

Animals, feeding, and treatments

Seventy-seven multiparous (4.3 ± 1.3 calving) Holstein cows with a body condition score (BCS) at calving of around 3 (2.95 ± 0.08) (1–5 scale; Edmonson et al. 1989) were included in this study. All cows grazed a mix of fresh pasture consisting of perennial ryegrass (*Lolium perenne*), kikuyo (*Cenchrus clandestinus*), annual ryegrass (*Lolium multiflorum*), white clover (*Trifolium repens*), and red clover (*Trifolium pratense*), which varied in different proportions according to farm and pastures where the cows grazed before and after calving. Cows were supplemented with 2 kg/day of concentrate from 30 days before calving, and with 4–6 kg/

day after calving; also, cows had free access to mineral supplementation and water. The chemical composition of the basal diet and the concentrate are shown in Table 1.

Clinically and gynecologically healthy cows that reached 42 to 35 days before calving were randomly assigned by the researchers to two groups: clinoptilolite-supplemented group (CLG, *n* = 42) and control group (CG, *n* = 35). Cows in each group were identified with different colored collars to prevent errors during clinoptilolite supplementation. The experiment was conducted from day 30 before the expected date of calving until day 60 postpartum. During prepartum period, CLG cows were supplemented with 50 g/cow/day of clinoptilolite (Captalin®; La Colina Cia. Ltda., Cuenca, Ecuador) (www.lacolina.com.ec) and after calving with 200 g/cow/day (following the manufacturer's recommendation). Clinoptilolite mixed with the concentrate was dispensed once a day in individual feeders, during milking. In order not to disturb the rotational grazing of the dairy herds, the cows under study were managed together with the other cows in production. Cows with dystocia at calving, retention of fetal membranes, purulent or mucopurulent vaginal discharge after calving were excluded; hence, only cow clinically healthy after calving were included in the current study. According to farm, cows were distributed as follows: farm A (*n* = 35), CLG 19 (parity, 4.3 ± 1.5; BCS, 2.86 ± 0.35) and CG 16 (parity: 4.4 ± 2.0; BCS: 2.88 ± 0.45) cows; farm B (*n* = 42), CLG 23 (parity, 4.2 ± 1.5; BCS, 3.08 ± 0.35) and CG 19 (parity: 4.3 ± 1.5; BCS: 3.0 ± 0.30) cows.

All animals were handled according to procedures approved by the Veterinary Medicine Career Committee, Faculty of Agricultural Science, University of Cuenca, Ecuador, and the research was conducted following chapter 7.8 of the Terrestrial Animal Health Code -2019© OIE (08/07/2019), regarding to protection of animals used in scientific experiments.

Table 1 Chemical composition of the grass and concentrate used in the diet of multiparous lactating dairy cow

	Diet composition (%)				
	DM	EE	CP	CF	NFE
Farm A					
Grass					
Dry period	12.3	2.3	19.2	27.9	34.4
Lactation	15.1	2.5	23.8	24.5	38.4
Concentrate	89.5	3.4	18.0	18.2	56.8
Farm B					
Grass					
Dry period	10.4	2.4	18.2	27.5	33.5
Lactation	15.5	2.5	23.6	23.9	38.4
Concentrate	89.1	3.4	17.5	17.6	54.5

DM dry matter; *EE* ethereal extract; *CP* crude protein; *CF* crude fiber; *NFE* nitrogen-free extract

Uterine cytology

Samples for endometrial cytology were collected in duplicate between days 35 and 45 (38.9 ± 3.6 days) after calving as described by Garzón et al. (2017). An adapted cytobrush to take uterine endometrial samples in cows was used. Briefly, a sterile cytobrush (CE-Model, ZY-H001, Caricia, Ecuador) was mounted in an artificial insemination (AI) gun and covered with a nylon sheath. After passing through the internal cervical os, the cytobrush was exteriorized, rotated onto the uterine mucosa, and reinserted into the AI gun cover. Once outside the cow, the cytobrush was smeared on a microscope slide, and each smear was air-dried and fixed with ethyl alcohol to preserve cellular morphology. Endometrial samples were stained with Wright® staining (JQWRG-1 K0-00; Quimical, Ecuador), and observed under a microscope (Olympus CX31; Germany) at × 400 magnification. Two hundred cells were counted to determine the proportion of PMN leukocytes in each cytology. Cows with ≥ 10% of polymorphonuclear leukocytes were considered to have subclinical endometritis, as described by Kasimanickam et al. (2004).

Ultrasonography

The ovaries of each cow were examined by ultrasound once a week for three consecutive weeks, starting at day 19.6 ± 3.6 after calving, with a portable ultrasound scanner (Draminski, iScan, Poland) and a 7.5-MHz linear array transductor. The diameters of all detected follicles, day of detection, and size of the first corpus luteum were recorded. Follicles were categorized according to size into three classes: < 5 mm (class A), 5–10 mm (class B), ≥ 10 mm (class C).

Reproductive variables

Following a voluntary waiting period of 45 to 60 days after calving, estrous detection was performed by visual observation for 1 h twice a day (06:00 to 07:00 h and 17:00 to 18:00 h). In cows in estrus, artificial insemination (AI) was performed according to AM-PM rule, using frozen-thawed commercial bull semen with proven fertility. Pregnancy diagnosis was performed by rectal examination 45 days after artificial insemination in cows with non-return to estrus by an experienced veterinarian. The AI dates and number of service per conception were recorded, and the following reproductive variables were calculated: intervals from calving to detection of the first corpus luteum (CL), to first service and to conception, number of services per conception, and first service conception rate. The interval calving to detection of first corpus luteum, regardless of whether the corpus luteum had formed days before its detection, was considered as indicative of the first postpartum ovulation. On each farm, the technician who performed the IA was blinded to each experimental group.

Statistical analysis

Variables showing a skewed distribution according to the Shapiro-Wilks test were *Log10* transformed. Intervals to first corpus luteum, to first service, and to conception, number of service per conception, and number and percentage of PMN (number of PMN leukocytes divide by total cell number × 100) were analyzed by general lineal model of SAS (V 9.3; SAS Institute, Inc., Cary, NC, USA). Effects of treatment, farm, parity, and treatment × farm interaction were included in the statistical model. Means were compared using least squares means test of SAS, and data were expressed as means ± SEM. Proportion of cows with subclinical endometritis, first service conception rate, and accumulated pregnancy at 45, 60, 75, and 90 days after calving were analyzed by chi-square test of SAS. Statistical significance was considered as $P<0.05$, and P values between 0.051 and 0.1 were considered as tendency.

Results

Body condition decreased as the postpartum days increased, without significant differences between experimental groups during the first 36 days after calving (Table 2). Numbers of follicles in different classes were similar between groups at all three ultrasound evaluations, with the exception of 5–10 mm (class B) follicles, whose number was significantly greater at the second ultrasound assessment in CG cows ($P=0.0077$). The CLG group had the largest follicle at the first ($P=0.0325$) and third ($P=0.0282$) ultrasound examinations (Table 2).

Number and percentage of uterine PMN leukocytes were lower ($P<0.0001$) in CLG than in CG (Fig. 1). For number and percentage of uterine PMN leukocytes, there was no effect of farm ($P=0.9805$), parity ($P=0.4199$), or treatment × farm ($P=0.1498$). The adjusted means for these variables on farm A were 9.1 ± 1.8 (CLG) and 16.9 ± 1.9 (CG) PMN leukocytes ($P=0.0020$), and 4.4 ± 0.8 (CLG) and 8.0 ± 0.9% (CG) ($P=0.0025$) respectively; on farm B were 6.6 ± 1.8 (CLG) and 19.0 ± 1.9 (CG) PMN leukocytes ($P<0.0001$), and 3.2 ± 0.8 (CLG) and 9.2 ± 0.9% (CG) ($P<0.0001$), respectively.

The interval from calving to detection of first corpus luteum (FCL) was no affected by treatment ($P=0.0759$), farm ($P=0.6227$), parity ($P=0.4190$), or treatment × farm

Table 2 Body condition and follicular characteristics (mean ± SEM) in early postpartum in multiparous lactating dairy cows supplemented with clinoptilolite

Variables	Treatment		*P* value		
	Clinoptilolite ($n=42$)	Control ($n=35$)	T	F	T×F
Body condition at calving	2.92 ± 0.08	2.99 ± 0.08	0.4827	0.1027	0.3778
First ultrasound (days)	20.0 ± 0.6	19.3 ± 0.5	-	-	-
Body condition	2.84 ± 0.06	2.88 ± 0.06	0.5707	0.3791	0.7702
Class A follicles (n)	7.7 ± 2.0	10.2 ± 1.8	0.2440	0.0254	0.6993
Class B follicles (n)	1.32 ± 0.44	1.40 ± 0.40	0.7754	0.0034	0.9684
Class C follicles (n)	1.20 ± 0.21	1.15 ± 0.19	0.9886	0.3714	0.8793
Largest follicle (mm)	14.2 ± 1.3	11.3 ± 1.2	0.0325	0.7896	0.3150
Second ultrasound (days)	28.1 ± 0.7	27.6 ± 0.6	-	-	-
Body condition	2.82 ± 0.07	2.81 ± 0.07	0.9097	0.6799	0.6847
Class A follicles (n)	7.8 ± 1.3	7.3 ± 1.4	0.8661	0.4538	0.8275
Class B follicles (n)	0.98 ± 0.24	1.90 ± 0.26	0.0077	0.2506	0.3551
Class C follicles (n)	1.17 ± 0.16	1.05 ± 0.16	0.9001	0.1813	0.9172
Largest follicle (mm)	12.5 ± 1.2	11.4 ± 1.3	0.4897	0.7153	0.9248
Third ultrasound (days)	34.1 ± 0.6	35.6 ± 0.9	-	-	-
Body condition	2.80 ± 0.07	2.68 ± 0.08	0.1874	0.3881	0.5306
Class A follicles (n)	8.0 ± 1.9	8.0 ± 1.8	0.8416	0.5881	0.7829
Class B follicles (n)	1.03 ± 0.33	0.98 ± 0.32	0.5767	0.0357	0.1259
Class C follicles (n)	0.97 ± 0.17	0.73 ± 0.16	0.2865	0.4060	0.1947
Largest follicle (mm)	14.7 ± 1.5	10.2 ± 1.5	0.0282	0.2489	0.6884

T treatment; *F* farm; *T×F* treatment × farm. Follicle categories: class A, ≤ 5 mm; class B, > 5 < 10 mm; class C, > 10 mm

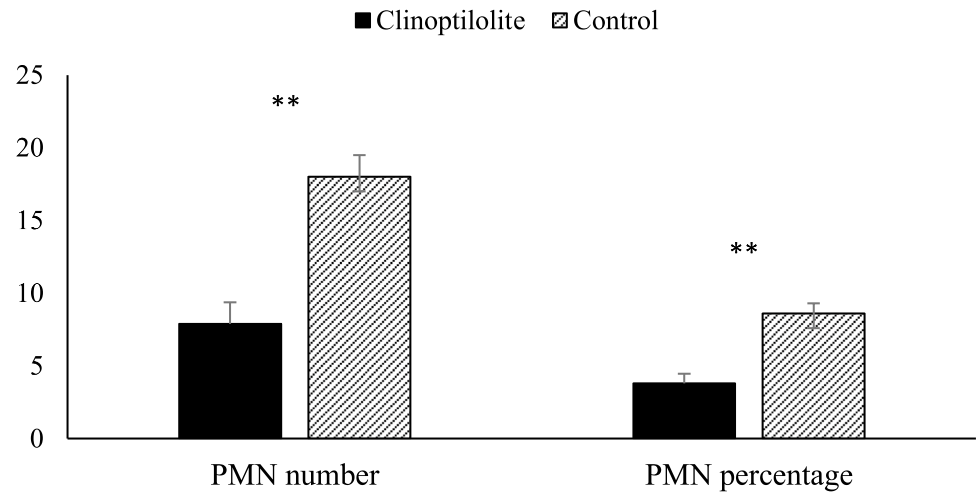

Fig. 1 Content of uterine polymorphonuclear leukocytes (number and percentage) in the early postpartum of multiparous lactating cows according to whether or not they were supplemented with clinoptilolite. $^{**}P<0.0001$

($P = 0.0743$). This interval was 3.5 days shorter in CLG than CG (Table 3). On farm A, FCL interval was 25.7 ± 1.9 and 25.7 ± 2.2 days for CLG and CG ($P = 0.9959$), respectively. On farm B, FCL interval was 21.2 ± 1.9 and 28.3 ± 2.1 days for CLG and CG ($P = 0.0115$), respectively. Corpus luteum diameter was not affected by treatment ($P = 0.3258$), farm ($P = 0.5963$), parity ($P = 0.6750$), or treatment × farm ($P = 0.8708$).

Calving to first service interval was no affected by treatment ($P = 0.6750$), farm ($P = 0.3032$), parity ($P = 0.1345$), or treatment × farm ($P = 0.9937$). However, calving to conception interval was 35 days earlier in CLG than CG cows (Table 3). There was an effect of treatment ($P = 0.0224$) and treatment × farm ($P = 0.0010$), but not of farm ($P = 0.0878$) or parity ($P = 0.7432$) on this reproductive variable. On farm A, calving to conception interval was 18.1 days longer in CLG than in CG (123.3 ± 14.6 v 105.2 ± 15.7 days, respectively; $P = 0.3750$), whereas in farm B this interval was 86.2 days shorter in CLG than in CG (96.5 ± 16.1 v 182.7 ± 17.5 days, respectively; $P = 0.0002$).

Number of services per conception (SC) was no affected by treatment ($P = 0.1882$) (Table 3), farm ($P = 0.9177$), parity ($P = 0.1593$), or treatment × farm ($P = 0.0831$). On farm A, SC was 1.67 ± 0.22 and 1.56 ± 0.24 IA for CLG and CG ($P = 0.7324$), respectively. On farm B, SC was 1.29 ± 0.24 and 1.99 ± 0.27 IA for CLG and CG ($P = 0.0396$), respectively. Cows with a CL at day 30 after calving was 11.9 percentage point greater in CLG than in CG ($P = 0.2982$). On farm A, cows with a CL at day 30 after calving was 57.9 and 50% for CLG and CG ($P = 0.6405$), whereas on farm B, these values were 52.2 and 36.8% for CLG and CG ($P = 0.3204$).

First service conception rate (FSC) was 6.8 percentage point greater in CLG and CG ($P = 0.6019$). On farm A, FSC was 39.0 and 61.5% for CLG and CG ($P = 0.2131$), respectively. On farm B, this reproductive index was 81.2

Table 3 Postpartum intervals, services per conception, corpus luteum diameter, first service conception, and proportion of multiparous lactating dairy cows with a corpus luteum at day 30 postpartum, and with subclinical endometritis according to whether or not they were supplemented with clinoptilolite

Variables	Treatment			
	Clinoptilolite ($n = 42$)		Control ($n = 35$)	
	n	Mean ± SEM or percentage	*n*	Mean ± SEM or percentage
Postpartum intervals to (days)				
1st corpus luteum	27	23.5 ± 1.6^{a}	23	27.0 ± 1.6^{b}
First service	42	76.6 ± 6.3	34	75.4 ± 6.2
Conception	34	109.9 ± 12.0^{b}	25	144.0 ± 12.4^{c}
Number of services per conception	34	1.48 ± 0.19	25	1.78 ± 0.18
Corpus luteum diameter (mm)	27	25.9 ± 1.5	23	24.0 ± 1.6
Cows with a CL at day 30 (%) *	23	54.7	15	42.8
First service conception (%)	20	58.8	13	52.0
Cows with subclinical endometritis (%)	2	4.7^{c}	8	22.8^{d}

Values with different letters in the same column differ. $^{a\text{–}b}$ $P = 0.0759$; $^{b\text{–}c}$ $P = 0.0224$; $^{c\text{–}d}$ $P < 0.0001$. * Proportion of cows with a corpus luteum at day 30 postpartum and 41.7% for CLG and CG ($P = 0.0305$), respectively. Figure 2 shows the progression of cumulative pregnancy up to 90 days postpartum by treatment. At day 90 postpartum, 37.7% (15/42) of cows were pregnant in CLG and 20.0% (7/35) in CG ($P = 0.1285$). On farm A, rate of pregnancy 90 days after calving was 21.0% (4/19) for CLG and 43.7% (7/16) for CG ($P = 0.1496$). On farm B, this reproductive variable was 47.8% (11/23) for CLG and 0% (0/19) for CG ($P = 0.0005$).

Proportion of cows with subclinical endometritis was significantly lower ($P = 0.0187$) in CLG than in CG (Table 3). On farm A, 10.5% (2/19) of cows had subclinical endometritis in CLG and 25.0% (4/16) in CG ($P = 0.2577$). On farm B, subclinical endometritis affected 0% (0/23) of cows in CLG and 21.0% (4/19) in CG ($P = 0.0207$).

After 1 year of study, there were 19.0% (8/42) of open cows in CLG and 28.5% (10/35) in CG ($P = 0.3255$). On farm A, 5.2% (1/19) (CLG) and 31.2% (5/16) (CG) of cows were non-pregnant ($P = 0.2116$), while on farm B, open cows were 26.3% (5/19) and 30.4% (7/23) for CLG and CG, respectively ($P = 0.6611$).

Discussion

This study aimed to assess the effect of supplementing clinoptilolite to multiparous dairy cows managed in a mountain tropical pasture-based system on reproductive performance and uterine health. Overall analysis showed that the addition of clinoptilolite to the diet significantly reduced the interval from calving to conception, the percentage of uterine PMN leukocytes, and the proportion of cows with subclinical endometritis. Clinoptilolite may have improved uterine health and reduced the interval to conception through two mechanisms: (1) modifying ruminal physiology (Urías-Estrada et al. 2017) and as a consequence improving the metabolic status (Karatzia et al. 2013; Folnožić et al. 2019) and (2) strengthening the immune system of cows (Ural 2014; Đuričić et al. 2017, 2020a, b).

However, it is important to note that on farm A reproductive performance was statistically similar between treatments, while on farm B the positive effect of clinoptilolite was quite pronounced. In the experimental conditions of this field study, it was not possible to know the specific causes of this discrepancy. Although both farms had similar general management (based on grazing, concentrate supplementation during milking, twice daily mechanical milking), some conditions, such as the altitude, were different (farm A is about 450 higher than farm B). At distinct altitudes, different climatic factors or proportions of grasses and legumes in the pastures may have been present. In this context, some uncontrolled conditions likely interacted to determine different animal responses to clinoptilolite.

In the current study, body condition did not differ between groups in the first 35 days postpartum, indicating that addition of clinoptilolite in the diet did not improve the energy balance of cows in the early postpartum period. In a previous study, a greater dose of clinoptilolite (180 and 270 g/cow/day during 60 days before and 45 days after calving, respectively) supplemented to the basal diet improved body condition score in multiparous lactating dairy cows managed under similar conditions to the current study (Garzón et al. 2017). In another study, clinoptilolite supplementation (200 g daily) increased body condition score and blood serum concentration of glucose and acetoacetate, and decreased blood serum concentration of ketone bodies, without affecting milk production (Karatzia et al. 2013). However, both studies were conducted

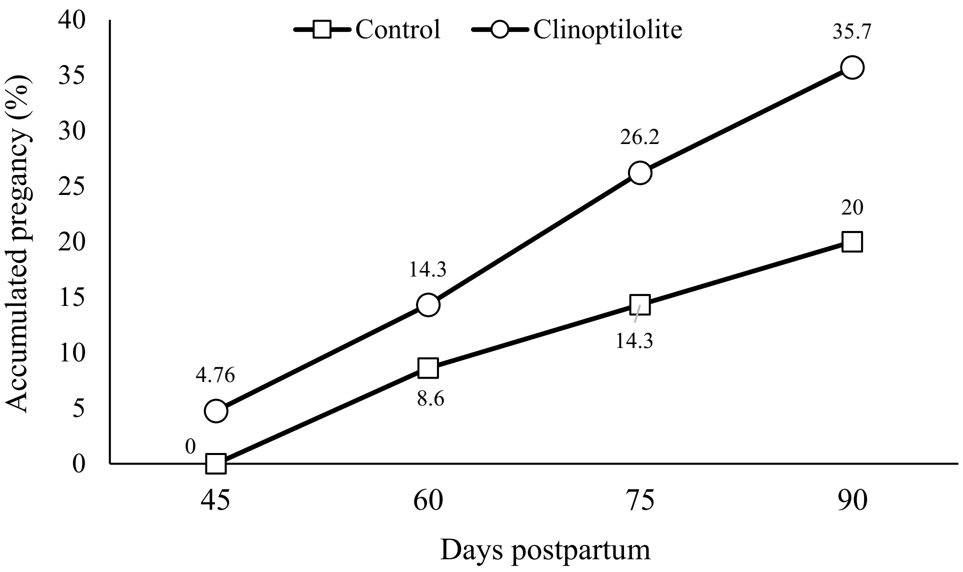

Fig. 2 Accumulated pregnancy at 45, 60, 75, and 90 days postpartum in multiparous lactating dairy cows according to whether or not they were supplemented with clinoptilolite

in animals (cows and heifers, respectively) in which supplementation with clinoptilolite began between 210 to 225 days of gestation, and exhibited a considerably better body condition at calving.

Rapid resumption of cyclic ovarian activity after calving is essential to achieve maximum productivity in the dairy herd (Giordano et al. 2012). In this study, resumption of ovarian cyclicity determined by detection of a corpus luteum by ultrasound in the first 35 days after calving was slightly faster in CLG than CG, although diameter of luteal tissue did not vary between groups. As a reference, proportion of cows with a corpus luteum at day 30 after calving was around 12 percentage points greater in CLG than in CG ($P = 0.2982$). No published data were found on the effect of the addition of clinoptilolite in the basal diet on resumption of ovarian cyclicity, as determined in the present study. However, supplementation of dairy heifers with 200 g of clinoptilolite reduced the interval to the first postpartum estrous by 6 days (Karatzia et al. 2013). Based on the detection of a follicle ≥ 10 mm as indicative of resumption of ovarian activity, Garzón et al. (2017) found that supplementation of multiparous dairy cows with clinoptilolite (180 and 270 g/cow/day during the dry period and early postpartum, respectively) doubled the proportion of cows that returned to ovarian activity within a period of time 45 days after calving.

In the aforementioned studies, the supplementation of greater amounts of clinoptilolite began at least 60 days before the expected date of calving, whereas in our study it began 30 days before parturition with 50 g/cow/day of clinoptilolite. The addition of clinoptilolite prior to parturition was intended to promote the adaptation of this additive in the rumen. The considerably lower amount added to the diet during this period in the current study may explain the lack of effect on body condition and on resumption of reproductive cyclicity and calving to first service interval. It is reasonable that adding a larger amount of clinoptilolite over a longer period before parturition may have an effect on specific aspects of the body physiology leading to an energy balance favorable to reproductive function, as demonstrated by Karatzia et al. (2013) and Garzón et al. (2017). It is well known that a positive energy balance and a suitable postpartum body condition influence reproductive performance (Spicer et al. 1990; Butler and Smith 1989). Specific traits include increased circulating concentrations of progesterone, estradiol, and LH, enhanced number and quality of oocytes retrieved by ultrasound-guided transvaginal aspiration (Kendrick et al. 2010), reduced intervals to first estrus, and increased conception rate (Beam and Butler 1999; Butler 2003).

Addition of clinoptilolite significantly decreased the number and percentage of uterine PMN leukocytes. In a similar study in the southern Ecuadorian Andes (Garzón et al. 2017), cows supplemented with clinoptilolite had a significantly lower percentage (3.4%) of PMN leukocytes than the control group (22.3%), which agrees with findings in this study. Moreover, clinoptilolite significantly reduced the proportion of cows with subclinical endometritis, a finding not previously reported in cows. In a different experimental context, addition of clinoptilolite to lactating dairy cows decreased somatic cell count (Ural 2014), and numbers of bacterial causing mastitis, as well as the risk of intramammary infection compared to control group (Đuričić et al. 2017, 2020a, b).

Bacteria colonizing the uterus during and immediately after calving, many of them pathogenic (Williams et al. 2007), adhere to endometrium, and even penetrate deeper layers, causing varying degrees of infertility (Lazzari et al. 2011). Likewise, pathogenic bacteria produce endotoxins that affect reproductive performance of cows in various ways (Suzuki et al. 2001; Williams et al. 2001, 2008; Lavon et al. 2008), several of them related to the production of inflammatory mediators (Williams et al. 2007).

Clinoptilolite has been shown to have antibacterial, antioxidant, and anti-inflammatory and immunomodulatory effects in various species of mammals (review by Valpotić et al. 2017; Pavelić et al. 2018; Mastinu et al. 2019). For instance, 5 g daily of clinoptilolite from 180 days before calving to 60 days postpartum decreased haptoglobin and serum amyloid A, two antioxidative biomarkers (Folnožić et al. 2019). In mice, adding clinoptilolite in the food ration for 5 months reduced cell death induced by reactive oxygen species (ROS) and production of ROS in mitochondria (Montinaro et al. 2013). In weaned pigs, supplementing clinoptilolite in the diet reduced total bacteria in the jejunum and diarrhea severity and increased recruitment of $CD54RA^+$ cells (Valpotić et al. 2016). Addition of 200 g of clinoptilolite for 70 days in cows (*E. coli*–vaccinated on day 210–240 of gestation) increased specific antibody titers against *E. coli* in their serum and colostrum and in the serum of their calves (Karatzia 2010). Feeding newborn calves with 3 l of maternal colostrum containing 5 g/l of clinoptilolite caused 50% greater IgG levels at 24 and 48 h after birth than control calves (Fratric et al. 2005). In poultry, addition of 2 or 3% of clinoptilolite in the diet (for 40 days) to broiler chicks increased percentages of T lymphocytes expressing CD4 + CD25 +, and B lymphocytes expressing BU-1 + and MHC Class II + molecules, and in the CD4:CD8 ratio (Jarosz et al. 2017). Although the precise mechanism by means clinoptilolite increases immune activity in animals is unknown yet, this body of evidence clearly demonstrates the immune-stimulating property of this compound.

In light of the experimental evidence cited above, and results of the current study, it is likely that cows on CG diet had a weaker immune system, and as a consequence, had a greater number and percentage of uterine PMN leukocytes. Therefore, more of them had percentages of uterine PMN leukocytes indicative of subclinical endometritis.

According to evidence discussed above, it is plausible that the better reproductive performance achieved by CLG cows occurred due to the antioxidant and immune-stimulating properties of clinoptilolite. As a consequence, a healthier uterine environment favored clinoptilolite-supplemented cows to become pregnant earlier, with a first service conception rate 7 percentage points greater and 0.3 fewer inseminations than CG group. Moreover, CLG cows reached an accumulated pregnancy at 90 days postpartum 15.7 percentage points greater than CG. Ninety days postpartum is a critical time by which the reproductive goal of one calf and one lactation per year is attained (Perea et al. 2002). Each additional day to achieve pregnancy has a significant economic cost for dairy farms (De Vries 2006). Thus, addition of clinoptilolite to the feed ration from prepartum and during postpartum may be implemented as a routine strategy to improve uterine health and reproductive efficiency in dairy farms.

In conclusion, general results of this study showed that the daily addition of clinoptilolite to the diet before calving (50 g daily) and up to 60 days postpartum (200 g daily) had beneficial effects on reproduction by reducing the prevalence of subclinical endometritis and open days in multiparous lactating dairy cows managed in a mountain tropical pasture-based system. In addition, clinoptilolite had different effects on both farms under study. On farm A, most of the reproductive variables were statistically similar between experimental groups, whereas on farm B, clinoptilolite improve significantly general reproductive performance. In both farms clinoptilolite significantly reduced the content of uterine PMN leukocytes in the early postpartum period.

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Author contribution JG, AG, and GL conceived and designed research. FS and JZ conducted experiment and wrote original draft. GC and FQ contributed new reagents or analytical tools. FP analyzed data and reviewed and edited the manuscript.

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Data availability Data will be made available on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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