Effect of the administration of a ruminal bolus containing 1,25-dihydroxyvitamin D_3 glycosides from *Solanum Glaucophyllum* on blood calcium levels, hypocalcaemia, production performance, health status and fertility rate in lactating dairy cows

SILVIA GROSSI*[1], RICCARDO COMPIANI[2], GIANLUCA BALDI[3], KATHRIN BÜHLER[4], CARLO ANGELO SGOIFO ROSSI[1]

[1] University of Milan, Faculty of Veterinary Medicine and Animal Science, Via dell'Università 6, Lodi, 26900, Italy
[2] Doctor of Veterinary Medicine
[3] PhD - Animal Nutrition and Food Safety
[4] Herbonis Animal Health GmbH, Rheinstrasse 30, 4302 Augst, Switzerland

SUMMARY

The aim of the study was to evaluate the efficacy of a bolus containing natural glycosides extracted from *Solanum glaucopyllum*, rich in 1,25-dihydroxyvitamin D_3 glycosides (1,25$(OH)_2D_3$), in the prevention of subclinical hypocalcaemia, and of its correlated health and production problems in transition dairy cows.

A total of 60 pregnant Holstein Friesian cows were balanced for parity and assigned to one of the two study groups: Control (15 cows) and Treatment (45 cows), receiving a bolus containing either 0 or 500 µg of 1,25$(OH)_2D_3$ extracted from *S. glaucophyllum*. Blood total (tCa) and ionized (Ca^{2+}) calcium, phosphorus (P) and magnesium (Mg) were evaluated at different time points. Data related to health status, production and reproductive efficiency were evaluated, both considering the two entire populations and separately for healthy and sick cows that undergone to retained placenta and metritis.

The treatment has led to a significant increase in the blood tCa (2.26 mmol/L) and Ca^{2+} (2.19 mmol/L) at calving, compared to prepartum (respectively 2.05 and 1.98 mmol/L), while in the Control there was a significant decrease in both blood tCa and Ca^{2+} (respectively, 1.76 mmol/L and 1.74 mmol/L at calving *vs* 2.12 and 2.02 mmol/L prepartum) ($P<0.05$), with also a significant difference at calving between the two groups ($P<0.05$). No significant differences were found in terms of Mg and P concentrations. The treatment has led to a significantly lower incidence of subclinical hypocalcaemia at calving (13.33% *vs* 80.00% considering tCa, and 24.44% vs 86.67% considering Ca^{2+}) ($P<0.0001$) and after 24h (35.56% *vs* 73.33% considering tCa) ($P=0.009$). The treatment increased significantly Ca^{2+} levels at calving (1.95 *vs* 1.56 mmol/L) ($P<0.05$) in sick cows. The incidence of metritis was significantly reduced by the treatment (2.22 *vs* 20.00%) ($P<0.05$).

Milk production (27.93 *vs* 25.70 L/head/day), dry matter intake (DMI) (19.11 *vs* 18.30 kg/head/d dry matter) and feed conversion rate (FCR) (1.46 *vs* 1.40), in the first 15 days of lactation (DIM), and days to first heat (36.55 *vs* 41.86 days) and day to pregnancy (97.51 *vs* 107.47 days) were improved in treatment group compared to Control ($P<0.05$).

Similarly, also in sick animals, milk production (treatment 23.48 *vs* 19.22 L/head/d in Control), DMI (15.29 *vs* 13.86 kg dry matter/head/d) and FCR (1.53 *vs* 1.39), in the first 15 DIM, and days to first heat (53.00 *vs* 67.66 days), and days to pregnancy (125.50 *vs* 153.67 days) were improved by the treatment ($P<0.05$).

The results of the present study highlight a potential effectiveness of boluses containing 1,25$(OH)_2D_3$ extracted from *S. glaucophyllum* in reducing the incidence and severity of hypocalcaemia in transition dairy cows, with positive effect of both animal welfare and productivity.

KEY WORDS

Hypocalcaemia, dairy cows, retained placenta, milk fever, transition period.

INTRODUCTION

The transition period, from three weeks before to three weeks after calving, is the most challenging phase in the dairy cows farming system [1]. Sustaining the last stages of foetal development as well as the production of colostrum and the onset of lactation, required extensive endocrine, physiologic, immunological and metabolic alterations, exasperate also by the reduced dry matter intake, that lead to a concomitant negative energy and protein balance [1]. Failure to adapt to these changes may result in the development of clinical diseases in the postpartum period [2].

Specifically, strong changes occur in terms of calcium balance between the dry period and the onset of lactation. Indeed, dry

Corresponding Author:
Silvia Grossi (silvia-grossi1994@libero.it).

dairy cows need about 20 g of calcium per day, while the requirements reach 70g per day during the colostrum and early milk production [3]. Those increased needs, require improvements in the mechanisms of calcium absorption and release from the bone tissues, as well as a reduction in the urinary excretion [4]. Normocalcemic animals, with a correct calcium balance, usually have a calcium level greater or equal to 2.0 mmol/L [5]. Conversely, failure in the adaptation of those mechanisms led to the development of hypocalcaemia [6].

Hypocalcaemia is commonly classified as clinical or subclinical, basing on the presence or not of clinical symptoms, and on blood calcium levels [5,7,8]. Clinical and subclinical hypocalcaemia are common issues in the dairy industry, respectively with an incidence of 1-10% [4] and more than 25% for primiparous and 50% for multiparous [3,5]. Clinical cases are characterized by the typical signs of milk fever such as paresis, difficultness in stand up, in maintaining the standing position, and tremors accompanied also by systemic symptoms such as loss of appetite, nervousness and fever, together with a blood calcium level lower than 1.4 mmol/L [5,9,10]. Conversely, subclinical hypocalcaemia can be defined when the calcium serum levels are below 2 mmol/L, usually within 24h post calving, without any other clear symptoms [5,11]. Even if threshold of <2mmol/L of the blood calcium levels is the most used in the current literature and practical approaches, there are some recent studies that underline that also higher threshold (2.15-2.2 mmol/L) can be indicators of subclinical hypocalcaemia, due to their association with negative health or production outcomes [8,12]. Indeed, if the negative effects of the clinical form on health and production efficiency are well-known, also the subclinical one is associated with a variety of negative sequelae and can be considered as the gateway for other common post-partum diseases [6,13,14]. It is well know that an inadequate concentration of calcium in the blood can impair both the smooth muscle contraction and the immune cell function, especially in terms of functionality of neutrophils. Those alterations predispose to various puerperal diseases such as dystocia, uterus prolapse, placental retention, metritis, mastitis and ketosis [5,15,16]. Moreover, a reduced muscle contractility can lead to impaired ruminal functionality and increased risk of abomasum displacement, further reducing the feed intake and consequently the energy balance [13]. Hypocalcaemia also reduces fertility and reproductive efficiency [17,18,19], and the contractility of the teat sphincter muscle, responsible for the closure of the teat orifice after milking, increasing the risk of mastitis [13].

Several strategies have been developed to prevent this health issue. At calving, calcium can be administered directly in the form of boluses, or even injection, to increase rapidly the blood calcium levels, especially in cows that are more at risk of milk fever, such as the multiparous ones [20]. Specific preventive nutritional strategies are applied also during the dry period and consist mainly in a restrictive use of calcium, together with a correct content of magnesium and vitamin D, and in the administration of anionic salt. Those two strategies are used to maintain and increase the responsiveness of bone cell receptors to parathyroid hormone (PTH) with the final aim to increase calcium absorption from the bones [21].

Also, vitamin D_3 is strongly involved in the calcium homeostasis. Thus, its integration is used to prevent milk fever and hypocalcaemia-related problems. However, vitamin D_3 *per se* is an inactive compound that, to be activated, must be converted to calcitriol (1,25-dihydroxyvitamin D_3 - 1,25$(OH)_2D_3$), through a calcium-dependent mechanism [22], but transition cows have a limited capacity to convert vitamin D_3 into its active form calcitriol (1,25$(OH)_2D_3$). In this meaning, the direct external supply of 1,25$(OH)_2D_3$, can be faster and more effective in preventing hypocalcaemia and milk fever. Since it is the final active metabolite of vitamin D and no further metabolism is required prior to acting on target cells in bone and intestine, the onset of action is more immediate and the potency greater than other metabolites [22]. Calcitriol increases circulating calcium levels by multiple mechanisms, upregulating the intestinal absorption and the osteoclasts formation and activity. In addition, it induces the reabsorption of calcium in the kidney distal tubules [23]. However, 1,25$(OH)_2D_3$ is not available as veterinary product, but it is contained in the *S. glaucophyllum* plant, in the form of glycosides [22]. The plant *S. glaucophyllum* can be administered directly, but delayed hypocalcaemia has occasionally been observed after the end of the administration, mainly due to an upregulation of the 1,25$(OH)_2D_3$-inactivating enzyme 24-hydroxylase [10,22].

Conversely, the glycosides from *S. glaucophyllum* can be extracted and condensed in specific products, such as boluses and tablets. Besides being easier to manage and administer to the cows, those products can modulate the speed of release of the 1,25$(OH)_2D_3$ compound, combining rapid and long-acting slow release of 1,25$(OH)_2D_3$ in the rumen. This combination ensures both a rapid increase in blood calcium levels able to counteract the calcium drop around calving, as well as a slow reduction of its efficacy, reducing the risk of delayed hypocalcaemia [18,19,22].

The aim of the present study was to evaluate the efficacy of the administration of a bolus containing natural glycosides extracted from *S. glaucopyllum*, rich in 1,25$(OH)_2D_3$, in the prevention of subclinical hypocalcaemia, and of its correlated health and production problems in Holstein dairy cows.

MATERIALS AND METHODS

2.1. Animals, diet and experimental design

The trial was carried out at the Del Santo farm, located in Castelgerundo (Lodi - Italy), with, on average, 350 lactating dairy cows, and management and structural characteristics that well reflects the typical situation of the intensive dairy farms.

A total of 60 pregnant dry Holstein Friesian dairy cows were balanced for parity and, in sequence to their expected calving date, assigned to one of the two study groups: (i) control with 15 cows (average lactation number of 2.2); (ii) treatment with 45 cows (average lactation number of 2.3).

Both the control and treated cows received a bolus composed of a capsule (8 cm * 2.4 cm) filled with dolomite as ballast and containing either 0 (Control) or 500 µg of 1,25$(OH)_2D_3$

Table 1 - Characteristics of the two different study groups: interval between bolus administration and calving.

Group	Interval bolus-calving, d
Treatment	4.5
Control	4.5

(Treatment) in the form of tablets containing *S. glaucophyllum* leaf extracts (SGE) (Herbonis Animal Health GmbH, August, Switzerland). The boluses were administered using a bolus applicator on average 5 days before the expected calving day.
Considering the natural variability in the calving timing and the optimal windows for SGE administration derived from pharmacokinetics studies [18], an interval between the bolus administration and calving of 3 to 6 days was allowed. The cows that did not calve within this interval were excluded from the trial and replaced. The registered interval between bolus administration and calving in the different groups were 4.5 d, All the animals were fed with the same dry and lactating diets (Table 2), formulated to meet or exceed the requirements for all nutrients, as reported by the NASEM [24].
The diets were administered *ad libitum* in the form of a total mixed ration (TMR) and distributed once a day in the morning through the use of a mixer wagon (Grizzly 71.26/2, capacity of 26 cubic meters mixing system with 2 vertical augers, Sgariboldi, Codogno, 2685 - LO, Italy), equipped with a balance, and designed to weigh both the inclusion of the individual ingredients and the unloaded TMR. Water was available *ad libitum*.
All the cows were housed together in a free-stall barn on straw bedding during the dry period and then, on average 5 days prior to parturition when the boluses were administered, moved to individual straw-bedded calving pens. The calves were separated from the cows right after calving. Two days after calving, the cows were moved to the lactation barn, on concrete floor with straw-bedded cubicles. All the cows were milked twice a day, in the morning at 07:00 and in the evening at 17:00, in a herringbone milking parlour that allows the simultaneous milking of 16 cows (8 + 8).

2.2. Sample collection and parameter recorded

The effects of the treatment were evaluated on blood parameters connected to hypocalcaemia as well as on production performances, fertility rate, health status and body condition score.

2.2.1. Blood parameters

Blood sample were taken at bolus administration (d-5), at calving (d0), at 24h (24h), 48h (48h) and 5 days (d5) after calving, from the jugular vein, using serum vacutainer without anticoagulant. The vacutainers were centrifuged, at room temperature, at 3500 g for 15 min right after the collection and the retained serum was stored frozen until analyses. Those serum samples were analysed for the total calcium (tCa), phosphorus (P) and magnesium (Mg) concentrations. Contextually, blood samples were anaerobically collected, for plasma ionized calcium (Ca^{2+}) analysis, in a disposable heparinized 2.5-ml syringe. Ca^{2+} concentrations were immediately determined using a blood pH gas-analyser (AVL Opti CCA, Idexx Italia, Milan, Italy). Conversely, tCa, P and Mg concentrations were analysed by an automatic spectrophotometer using o-Cresolphthalein complexone according to the manufacturer's instructions (BT3500 VET, Biotecnica Instrument, Rome, Italy). The threshold of 2 mmol/L of tCA and Ca^{2+} in the serum was used to evaluate the prevalence and risk of subclinical hypocalcaemia.

2.2.2. Production performances

Feed intake (kg/head/d) was recorded daily during the 7 days before calving and for the 15 days in milk (DIM), by weighting the TMR administered and the residue in the feed bunk after 24h hours. The feed intake was expressed as kg of dry mat-

Table 2 - Composition and nutritional values of the dry and fresh diets, as predicted by the rationing software (Plurimix).

Feed, kg/head/d as fed	Dry diet	Fresh diet
Corn silage 340731	10.00	22.50
Corn meal	-	5.00
Corn High Moisture with cobs 6055	-	3.00
Raygrass Silage 401050	2.00	3.00
Soybean meal 44% CP[1]	1.50	3.00
Alfaalfa Hay 1556	-	2.00
Raygrass hay	8.00	2.00
Linseed meal 33 CP	-	1.30
Rapeseed meal 33 CP	-	0.80
Mineral and Vitamins CalaMilk600	-	0.60
Mineral and Vitamins Dry and Heifers	0.15	-
	kg/head/d	
As fed, kg	*21.65*	*43.20*
DM[2], kg	*12.54*	*23.60*
	Analysis, % of DM. in the TMR	
DM, %	*57.91*	*54.62*
Energy, Mcal/ kg DM	*1.24*	*1.58*
UFL[3]/kg DM	*0.73*	*0.93*
CP	*12.51*	*15.40*
CF[4]	*1.86*	*2.93*
NDF[5]	*51.36*	*34.22*
ADF[6]	*29.33*	*20.03*
ADL[7]	*3.96*	*3.98*
Starch	*8.95*	*28.70*
Ca[8]	*0.63*	*0.64*
P[9]	*0.30*	*0.40*

[1]CP= crude protein; [2]DM= dry matter; [3]UFL= feed units for lactation; [4]CF= crude fats ;[5]NDF= neutral detergent fiber; [6]ADF= acid detergent fiber; [7]ADL= acid detergent lignin; [8]Ca= calcium; [9]P=phosphorus.

ter (d.m).

The daily milk yield (L/head/day) was recorded for each cow in the two groups for the first 15 days of lactation (DIM). The milk yield was recorded using a program, similar to the DairyComp programs, developed specifically for the farm by the Farm Computer System company (Cremona, Italy). Moreover, milk production was also evaluated monthly during the first 3 months of lactation, using the data collected by the Lombardy Regional Breeders Association (ARAL).

Milk quality was analysed monthly during the first 3 months of lactation in terms of fat, protein, and urea percentages, and somatic cell count. Milk quality analyses were performed by the ARAL laboratory, with the Milkoscan TM FT 6500 Plus instrument (Foss, Hillerød, Denmark) that employs the Fourier Transform Infrared Spectroscopy (FTIR) measuring principle.

2.2.3. Health status

The health status of each cow was checked daily by the farm veterinary and qualified staff.

The threshold of 2.0 mmol/L of tCa and Ca^{2+} in the serum at calving (d0) and at 24h (24h), was used to evaluate the prevalence and risk of subclinical hypocalcaemia.

Each hypocalcaemia-related problems as milk fever, retained placenta and metritis were recorded. Cases of milk fever were defined by the farm veterinary staff when the cows showed the typical clinical symptoms, in particular difficultness in stand up, in maintaining the standing position, and tremors. Retained placenta was defined as failure to expel spontaneously the placenta between 12h after calving and the veterinary staff has to intervene. Cases of metritis was defined as the presence of a fetid reddish to brownish uterine discharge, accompanied or not by systemic signs of illness in the first days after calving. Furthermore, each case of mastitis, lameness and other pathological issues were recorded in each group.

2.2.4. Body condition score

At the bolus administration, at 5 days after calving and at 30 days of lactation, each cow was individually evaluated for body condition score (BCS) by the farm veterinary staff, as proposed by Edmonson et al. (1989) and Ferguson et al. (1994), through a visual and tactile evaluation of body fat reserves using a 5-point scale with 0.25-point increments (1—very thin cow; 5—excessively fat cow) where 3 represents the average body condition [25,26]. The evaluation focused on the rump and loin.

2.2.5. Fertility rate

Individual data about days to first heat, days to first service, days to pregnancy, number of services to pregnancy and reproductive dysfunctions (ovarian cysts, metritis etc.) were recorded by the farm staff for all the cows involved in the trial.

2.3. Statistical analyses

Data analysis was conducted using SAS statistical software (SAS 9.4, SAS Cary NC).

Data distribution and homogeneity of variances was tested using PROC UNIVARIATE (SAS 9.4 SAS Institute Inc., Cary, NC). Data about blood parameters and production performances (milk yield, feed intake) were analyzed using a mixed model for repeated measures (PROC MIXED) which took into account the fixed effect of the treatment and the time of detection. The Tukey post-hoc test was applied to differentiate multiple comparisons. Blood parameters and feed intake and production performance, during the first 15 days of lactation, were also analysed separately for cows that undergone to health problems (retained placenta and metritis), to evaluate a potential efficacy of the Treatment in reducing the severity of those disease correlated with the calcium levels.

The incidence of subclinical hypocalcemia, highlighted by a blood tCa and Ca^{2+} than 2 mmol/L, was evaluated in the two study groups through a chi-square test (PROC FREQ)

The parameters related to fertility were evaluated using a mixed model (PROC MIXED) that took which took into account the fixed effect of the treatment. Reproductive parameters were also analyzed separately for healthy and sick cows that undergone to retained placenta and metritis disease correlated with the calcium levels. The BCS values were also evaluated, in terms of average values for each time point, using a mixed model (PROC MIXED) that took which took into account the fixed effect of the treatment. Non-continuous variables such as BCS distribution in the different thresholds, and incidence of pathologies, were evaluated through a chi-square test (PROC FREQ). The single subject was used as experimental unit in all the statistical evaluation. Statistical significance was considered with $P<0.05$.

RESULTS AND DISCUSSION

The trends in tCa and Ca^{2+} concentrations in the two study groups are reported in Figure 1 and 2. The trend in P and Mg concentration are reported in Figure 3 and 4. As visible in Figure 1 and 2, the treatment has led to significant differences in the Ca concentrations between the different time points, compared to the Control animals. In the Control group the tCa (1.76 mmol/L) and Ca^{2+} (1.74 mmol/L) levels significantly drop at calving (d0), compared to the prepartum levels (respectively 2.12 and 2.02 mmol/L) (d-5) ($P<0.05$). Conversely, in the Treatment group there was a significant increase in tCa (2.26 mmol/L) and Ca^{2+} (2.19 mmol/L) levels at calving (d0) compared to the prepartum levels (respectively 2.05 and 1.98 mmol/L) (d-5) ($P<0.05$). Consequently, at calving (d0) both tCa and Ca^{2+} levels were significantly higher in treated animals compared to controls ($P<0.05$). Those results are in line with the findings of Bachmann et al. (2017), that reported an increased serum Ca concentration around calving compared to the prepartum levels in cows treated with a bolus containing *S. glaucophyllum* extract, that was significantly higher compared to untreated cows [22]. Even if there was a significant drop in the tCa and Ca^{2+} levels in the first 24h after calving, in treated animals the levels were higher than the selected threshold of 2 mmol/L (respectively 2.05 and 2.01 mmol/L) ($P<0.05$), while in the Control group the threshold was reached only 48h after calving. Those data are in line with the findings of Meyer-Binzegger et al. (2022), that found a drop in Ca levels in the first 24h after calving also in animals treated with boluses containing *S. glaucophyllum* extract, but lower than in the control cows [19].

No significant differences were found in the trends and concentrations of both P and Mg between the two study groups. In both groups, the P levels increased at calving (d0) compared to pre-partum levels (d-5). After calving the P levels decreases continuously in both groups, without significant differences. A significant effect of time was detected in the Treatment group between calving (2.28 mmol/L) (d0) and five days of lactation

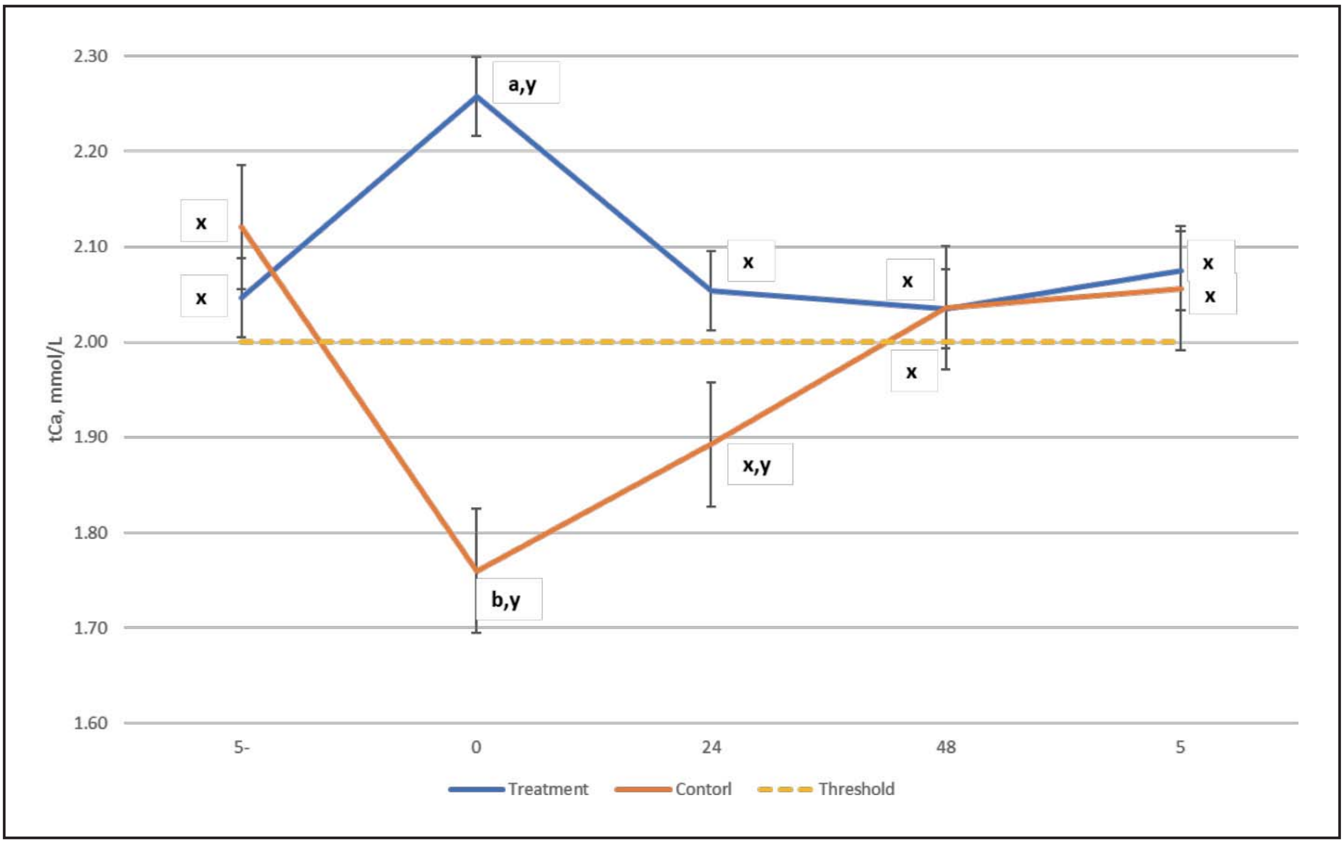

Figure 1 - Total calcium concentration, expressed as average values with standard errors, in Treatment vs Control cows. Different letter between different lines at same time point highlight significant differences (a, b P<0.05). Different letters in the same line (x, y) highlight significant differences between time points (x,y P<0.05).

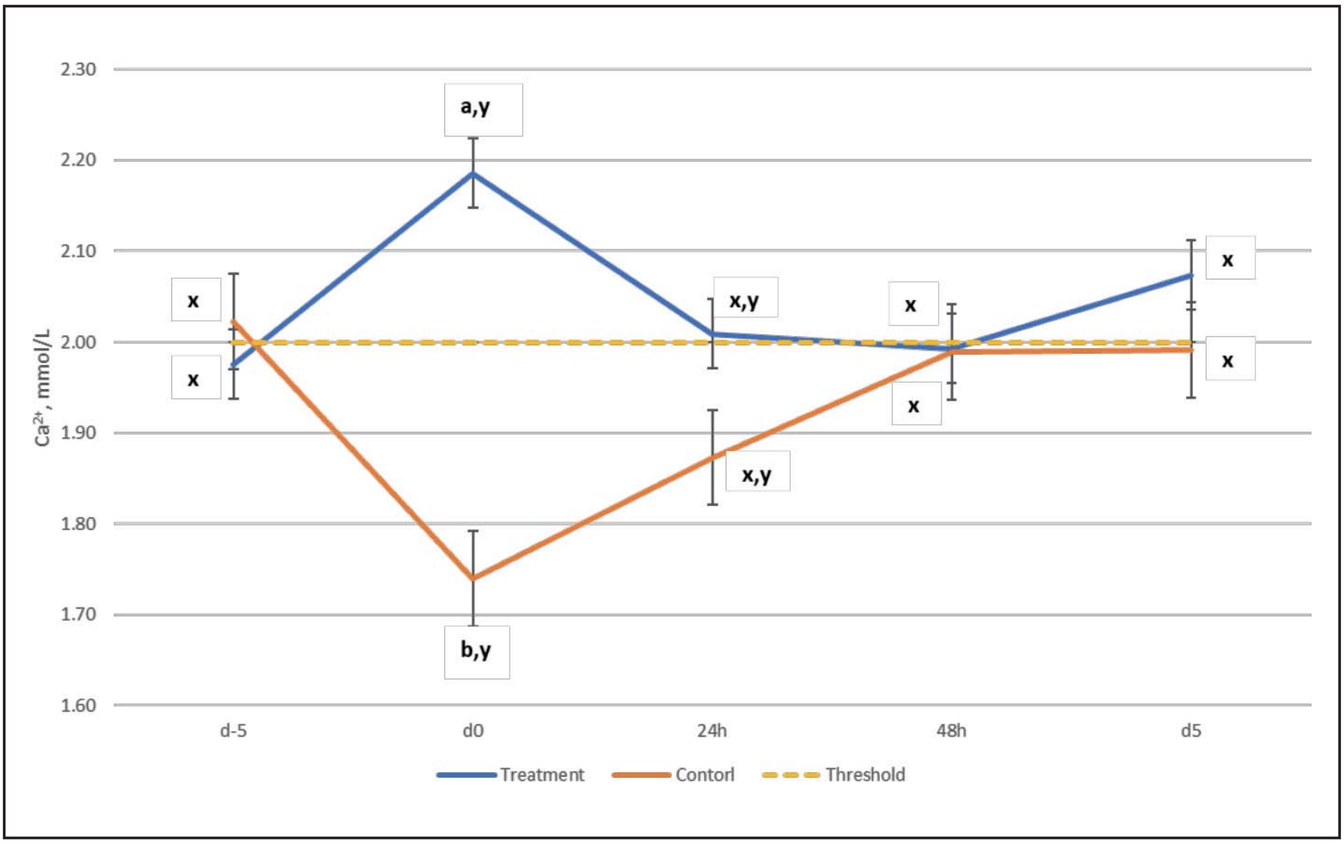

Figure 2 - Ionized calcium concentration in Treatment vs Control cows. Different letter between different lines at same time point highlight significant differences (a, b P<0.05). Different letters in the same line (x, y) highlight significant differences between time points (x,y P<0.05).

(2.04 mmol/L) (d5) (P<0.05). The increase in the P concentrations around parturition is in line with the findings of Bachmann et al. (2017) [22]. Conversely to the present study, Bachmann et al. (2017), found significantly higher P concentration in cows treated with *S. glaucophyllum* extract pre-partum compared to Control ones [22].

Considering the trend in Mg concentration, as visible in Figure 4, in both groups there was a significant drop at calving (d0), compared to the pre-partum levels (d-5) (respectively 0.931 *vs* 0.99 mmol/L in Treatment and 0.933 *vs* 0.98 mmol/L in Control) (P<0.05), while after calving the concentrations were stable. Conversely, Meyer-Binzegger et al. (2022), found that cows treated with bolus of *S. glaucophyllum* undergone to a lower Mg drop at calving and maintain a more stable Mg concentrations during the entire period compared to control animals [19]. Altogether, the average plasma Mg concentrations in both

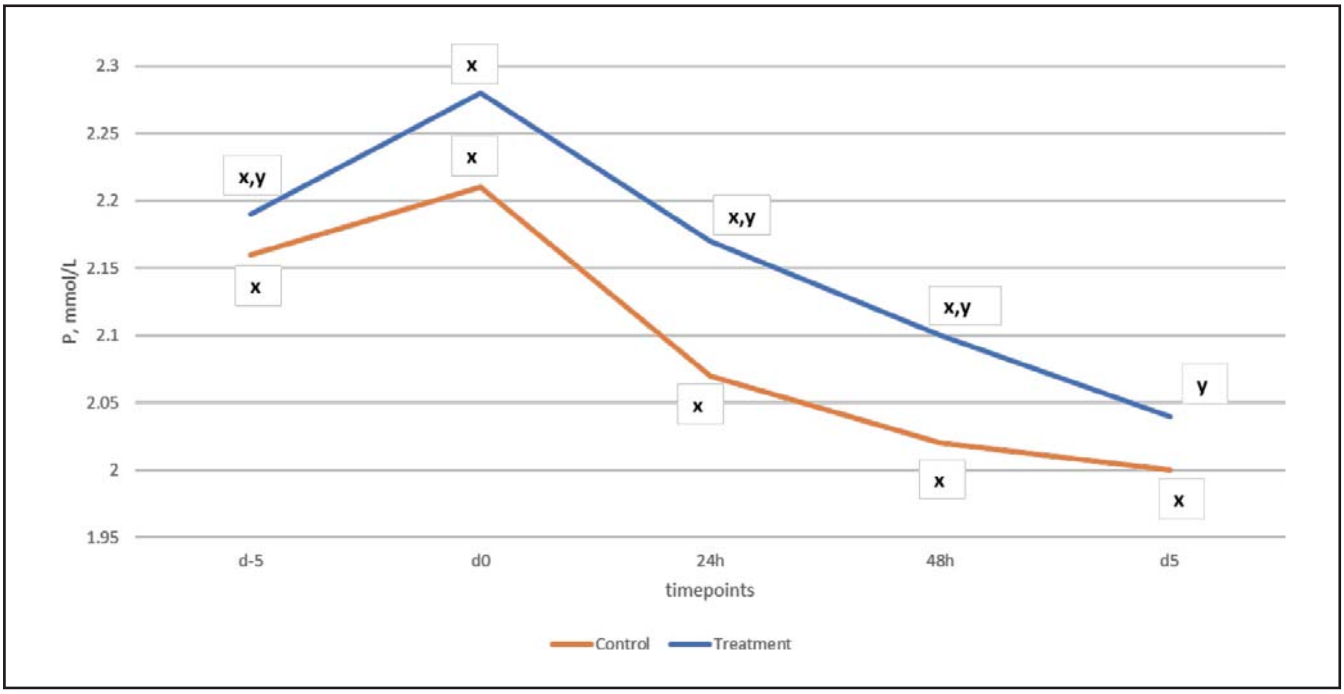

Figure 3 - Phosphorus concentrations in Treatment vs Control cows. No significant differences were found between the two groups (a, b P<0.05). Different letters in the same line (x, y) highlight significant differences between time points (x,y P<0.05).

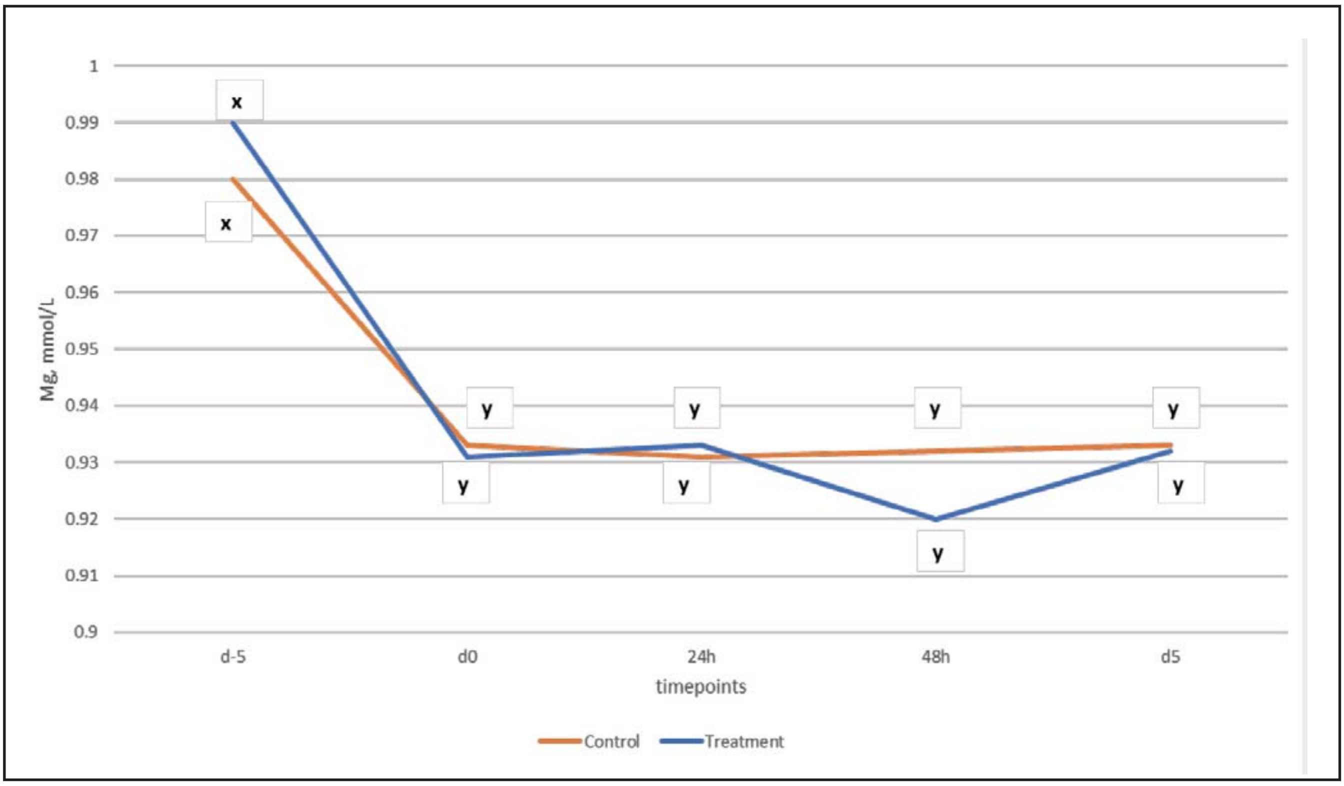

Figure 4 - Magnesium concentrations in Treatment vs Control cows. No significant differences were found between the two groups (a, b P<0.05). Different letters in the same line (x, y) highlight significant differences between time points (x,y P<0.05).

groups were in the normal range of normomagnesemia (between 0.75 and 1 mmol/L) [13].
In Figure 5 and 6 is reported the incidence of subclinical hypocalcaemia, expressed as the percentage of animals with blood Ca levels (tCa and Ca^{2+} respectively), lower than the threshold of 2 mmol/L, for each time points. Considering tCa, the treatment has led to a significantly lower incidence of subclinical hypocalcaemia at calving (13.33% *vs* 80.00% in Control) (P<0.0001) (d0) and after 24h (35.56% *vs* 73.33% in Control) (P=0.009) (24h). Considering Ca^{2+}, the treatment has led to a significantly lower incidence of subclinical hypocalcaemia at calving (24.44% vs 86.67% in Control) (P<0.0001) (d0).
The better Ca status in treated cows, together with the lower incidence of subclinical hypocalcaemia, has led to improvement in the overall health status and production parameters during

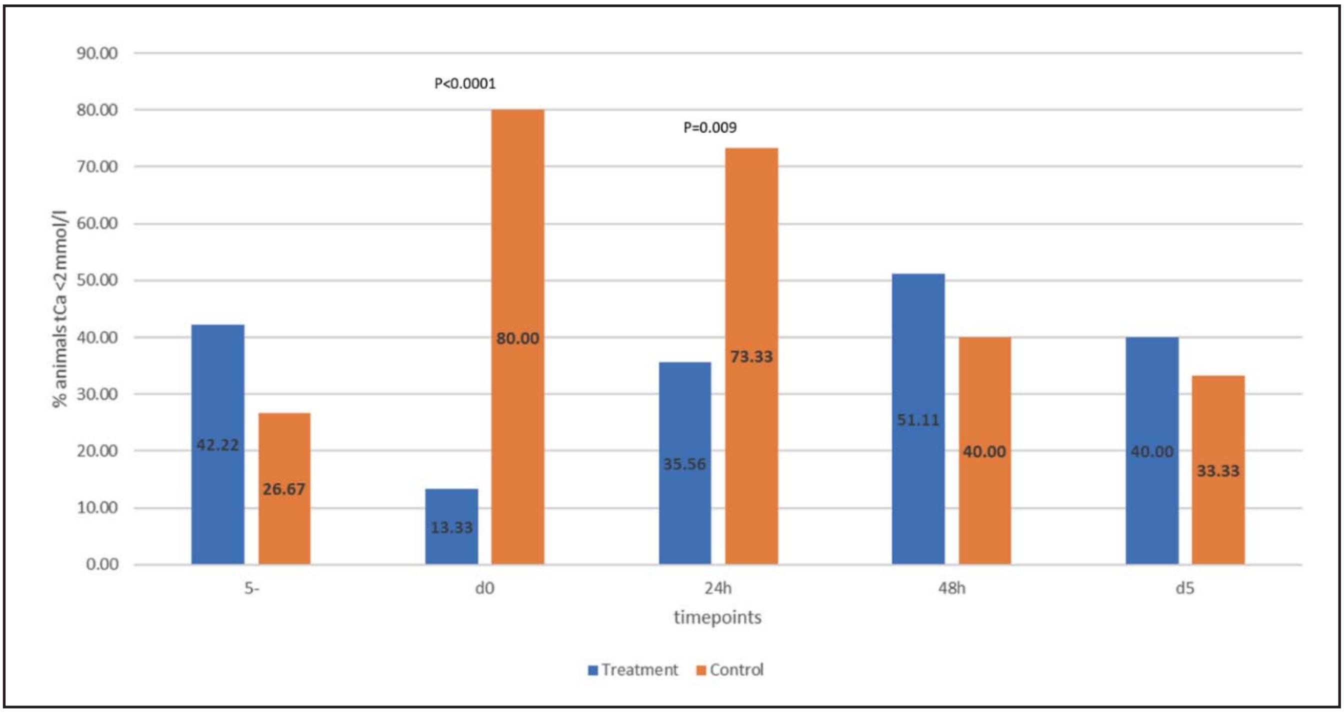

Figure 5 - Percentage of animals with total (tCa) calcium levels <2 mmol/L in the two study groups in the different time points

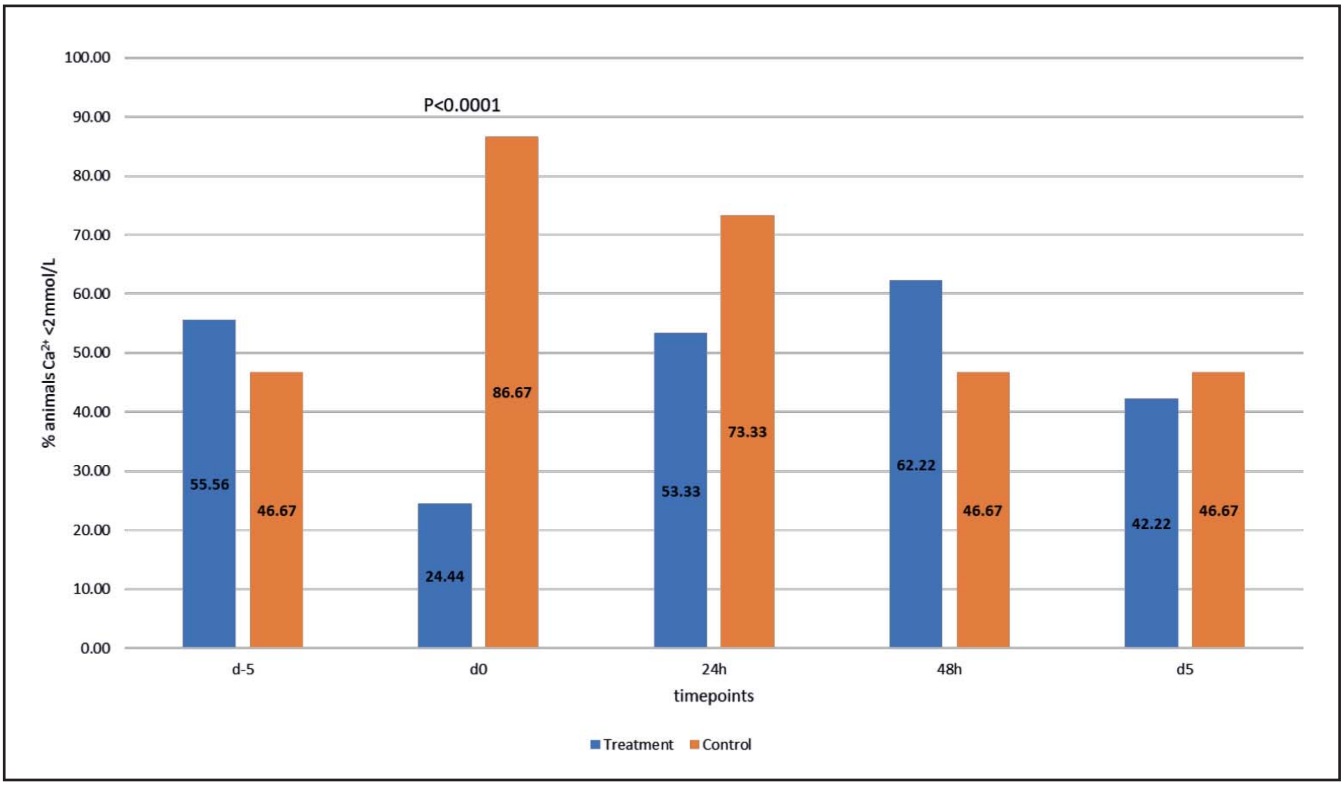

Figure 6 - Percentage of animals with ionized calcium (Ca2+) levels <2 mmol/L in the two study groups in the different time points

Table 3 - Health status in the two study groups.

Group	Retained placenta, % (n)	Clinical milk fever, % (n)	Metritis, % (n)	Mastitis, % (n)	Lameness, % (n)
Control	20.00 (3)	13.33 (2)	20.00 (3)	0.00 (0)	6.67 (1)
Treatment	8.89 (4)	4.44 (2)	2.22 (1)	2.22 (1)	8.89 (4)
P-value	ns	ns	<0.05	ns	ns

Table 4 - Ca^{2+} concentrations (mmol/L) in sick cows with retained placenta and metritis in the two study groups.

Ca^{2+}, mmol/L	Treatment	Control	*P*-Value
d-5[1]	1.77	1.86	ns
d0[2]	1.95	1.56	<0.05
24 h[3]	1.72	1.70	ns
48 h[4]	1.87	1.85	ns
d 5[5]	2.05	1.82	ns

[1]d-5= 5 days before calving; [2]d0=at calving; [3]24h= 24 hours after calving; [4]48h= 48 hours after calving; [5]d5= 5 days after calving

Table 5 - Feed intake in the last 7 days of the dry period and production parameters in the first 15 days in milk (DIM), considering the two entire study populations.

Parameter, unit	Treatment	Control	*P*-Value
Feed intake, last 7d of dry period	11.54 (± 0.76)[1]	11.51 (± 0.38)	ns
Milk yield, L/head/d	27.93 (±2.23)	25.70 (±3.48)	<0.05
Feed intake, kg d.m./head/d	19.11 (±1.85)	18.30(±2.24)	<0.05
Feed Efficiency, L/kg d.m.	1.46	1.40	<0.05

[1]results are present in terms of average values ± standard deviation

the first days of lactation.
As visible in Table 3, even if the differences were not statistically significant, the treatment has led to a numerically lower incidence of the two main pathologies related to the Ca levels, retained placenta (8.89 vs 20.00% in Control) and clinical milk fever (4.44 vs 13.33% in Control). Conversely, the incidence of metritis was significantly reduced by the treatment (2.22 *vs* 20.00%) (P<0.05).
The significantly higher blood Ca levels highlighted in treated animals can explain the lower incidence of milk fever but also retained placenta, due to an improved activity and reactivity of the uterine muscles, as well as due to an increased functionality of the immune systems [27]. Indeed, Rodriguez et al. (2017), found that the occurrence of retained placenta was 3.4 times higher in cows that had subclinical hypocalcaemia than in animals with normocalcaemia [15].
Moreover, in the present condition, the positive effect of the treatment on blood Ca levels was visible also in animals that undergone to retained placenta and metritis, as reported in Table 4. Indeed, sick animals in the Treatment group showed significantly higher Ca^{2+} levels at calving compared to sick animals in the Control group (1.95 *vs* 1.56 mmol/L) (P<0.05) and also a faster recovery of the Ca^{2+} levels. Specifically, in sick control cows the Ca^{2+} levels were still under the threshold of 2 mmol/L at 5 days after calving. Both higher Ca^{2+} levels and faster Ca^{2+} recovery in case of retained placenta, improved overall health status, production parameters and reproduction, lowering the risk of other correlated pathologies, like milk fever, metritis, ketosis, abomasal displacement and mastitis, improving also milk production efficiency [22].
In the present study, all the control cows with retained placenta have suffered from metritis with a statistically significant difference compared to treated cows (20.00% vs 2.22%, P<0.05). Youngquist and Threlfall (2007), reported that the risk of developing metritis is higher in cows that undergone to more severe cases of placenta retention, connected also to a slow recover of the normal blood calcium levels, as highlighted in sick control cows in the present conditions. Indeed, severe cases of retained placenta can cause an important inflammation response that predispose to metritis [28]. Rodriguez et al. (2017), highlighted a 4.3 times higher risk of developing metritis in cows with hypocalcaemia, due to an impaired functionality of the immune systems, especially of neutrophils [15].
Considering the two study populations, production efficiency resulted to be improved by the Treatment, as visible in Table 5. No statistically significant differences were found in the feed intake before calving, with also similar pattern in both groups, with the typical decrease during the last days before calving (Figure 7). Otherwise, after calving feed intake was significantly (P<0.05) increased by the Treatment (Table 6), of 0.82 kg d.m./head/d, corresponding to a 4.5% improvement compared to Control. After calving, cows undergo to a strong reduction in the feed intake, as a consequence of stress and physiological changes related to calving, that also lead to an important reduction in the immune functionality. An increase in the feed intake in the first days after calving, in addition to being a sign

Table 6 - Production parameters in the first 15 days in milk (DIM) in sick cows with retained placenta and metritis and in healthy cows in the two study groups

Parameter, unit	Treatment	Control	*P*-Value
Sick cows (retained placenta and metritis)			
Milk yield, L/head/d	23.48 (±2.31)[1]	19.22 (±0.46)	<0.05
Feed intake, kg d.m./head/d	15.29 (±0.34)	13.86 (±0.26)	<0.05
Feed Efficiency, L/kg d.m.	1.53	1.39	<0.05
Healthy cows			
Milk yield, L/head/d	28.37 (±1.64)	27.32 (±0.99)	<0.05
Feed intake, kg d.m./head/d	19.49 (±1.52)	19.41 (±1.51)	ns
Feed Efficiency, L/kg d.m.	1.45	1.41	ns

[1]results are present in terms of average values ± standard deviation

Table 7 - Monthly milk production in the first three months of lactation in the two study groups.

Milk, L/head/d	Treatment	Control	*P*-Value
First month	34.41 (±2.73)[1]	33.46 (±2.74)	ns
Second month	35.54 (±2.79)	34.67 (±2.78)	ns
Third month	35.70 (±2.42)	34.88 (±2.43)	ns

[1]results are present in terms of average values ± standard deviation

Table 8 - Milk quality parameters in the first three months of lactation in the two study groups

Month	Treatment	Control	*P*-Value
Fat, %			
First month	3.75 (±0.23)[1]	3.79 (±0.23)	ns
Second month	3.71 (±0.17)	3.77 (±0.17)	ns
Third month	3.77 (±0.14)	3.78 (±0.14)	ns
Protein, %			
First month	3.29 (±0.24)	3.27 (±0.24)	ns
Second month	3.35 (±0.19)	3.32 (±0.19)	ns
Third month	3.26 (±0.09)	3.25(±0.09)	ns
Caseins, %			
First month	2.81(±0.30)	2.82 (±0.30)	ns
Second month	2.80 (±0.22)	2.83 (±0.21)	ns
Third month	2.82 (±0.23)	2.84 (±0.23)	ns
Urea			
First month	22.06 (±1.79)	21.83 (±1.80)	ns
Second month	22.16 (±2.94)	22.30 (±2.95)	ns
Third month	22.49 (±1.74)	22.21 (±1.74)	ns
Somatic cells count			
First month	136.430 (±33.28)	118.688 (±33.21)	ns
Second month	138.711 (±33.18)	136.839 (±32.08)	ns
Third month	144.375 (±26.59)	140.174 (±22.95)	ns

[1]results are present in terms of average values ± standard deviation

Table 9 - Average body condition score (BCS) in the two experimental groups

Parameter	Treatment	Control	*P*-Value
5 days before calving	3.57	3.58	ns
5 days after calving	2.98	2.97	ns
30 days after calving	3.22	3.22	ns

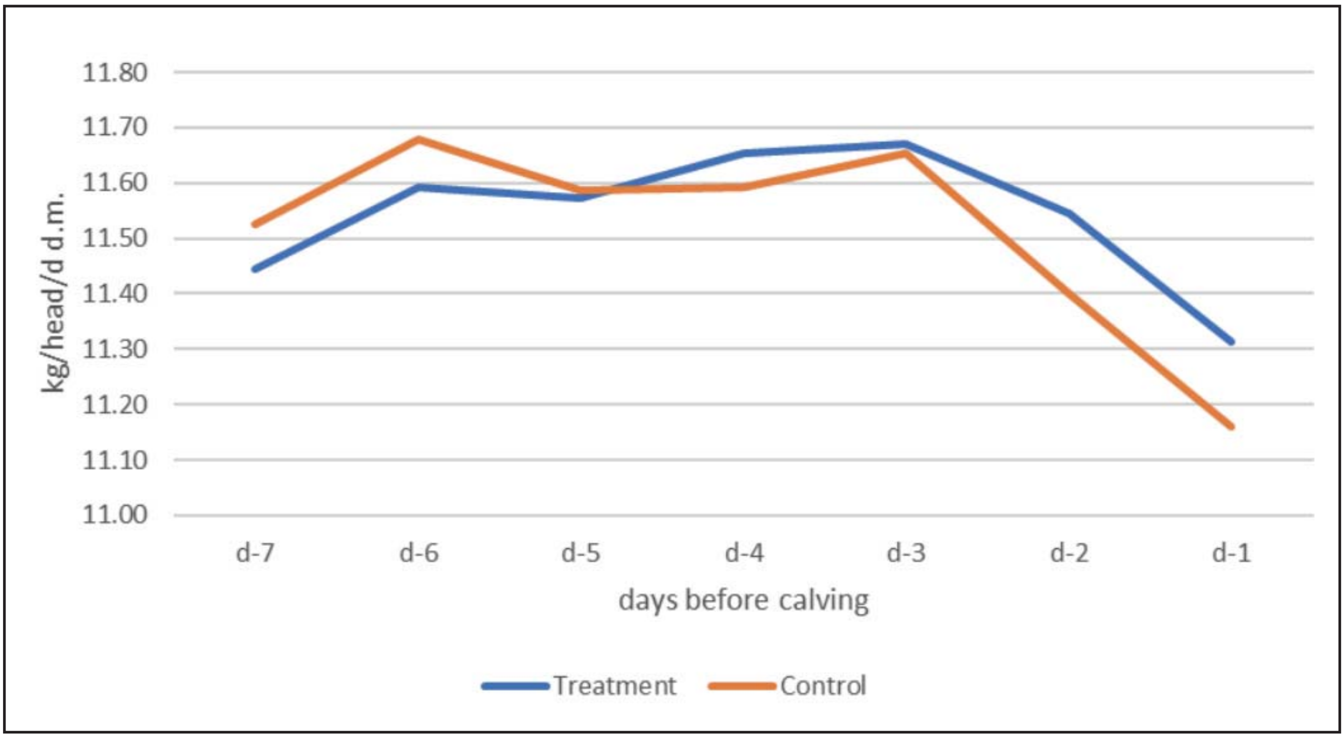

Figure 7 - Feed intake in the last 7 days of the dry period in the two groups.

of health, lead to a higher and faster capacity to respond to the stressful events, improving the health status. Moreover, a higher feed intake better support the increased energy and nutrients requirements at onset of lactation, reducing the body reserves mobilization and improving the metabolic status and reproductive activity in the first lactation phase. Indeed, in the present study, the treatment significantly ($P<0.05$) improved milk production during the first fifteen DIM by 2.23 L/head/day, corresponding to an increase of 8.68% compared to control cows. As shown in Figure 8, milk production started to differ from the third day DIM, with a faster and greater increase in treated cows. Those improvements can be ascribed to the re-

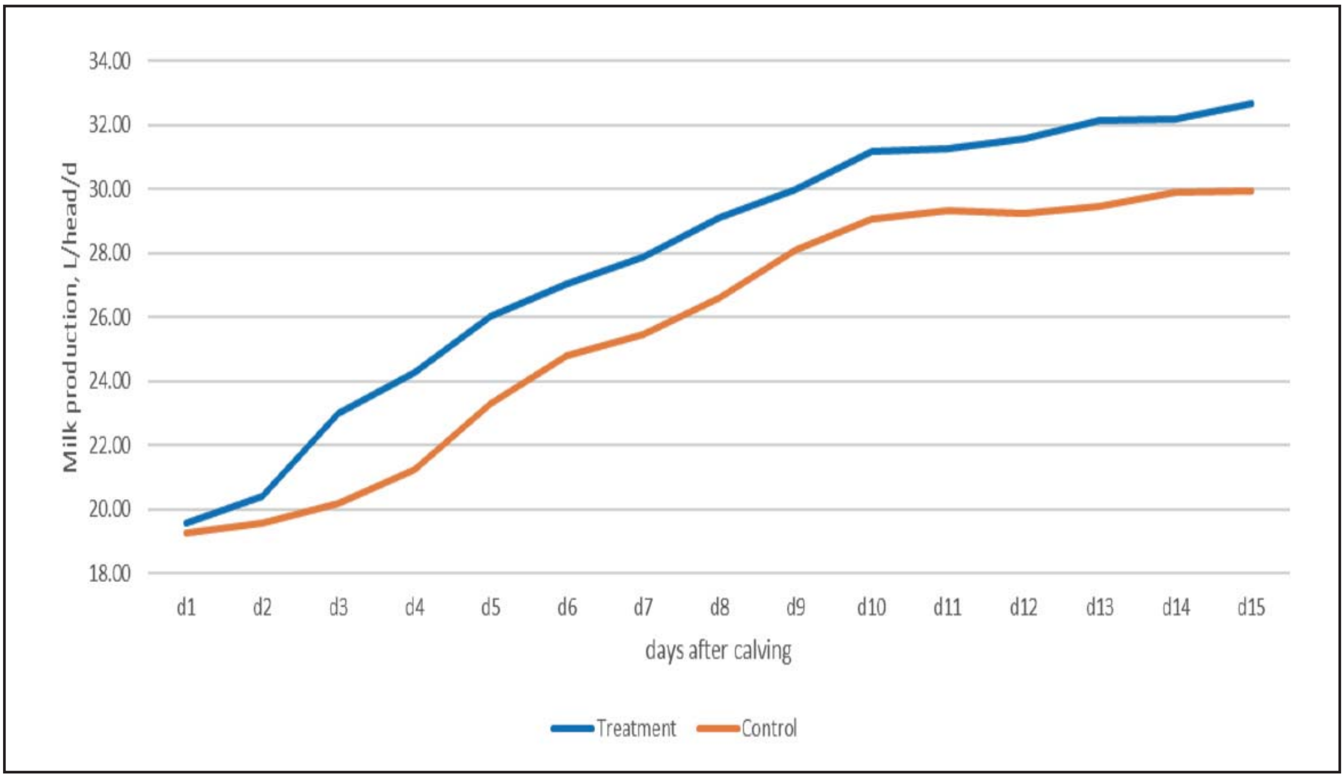

Figure 8 - Daily milk production in the first 15 days in milk production (DIM) in the two study groups.

duced incidence and severity of both subclinical hypocalcaemia and of clinical cases of retained placenta and metritis.

Indeed, milk production (23.48 *vs* 19.22 L/head/d), feed intake (15.29 *vs* 13.86 kg/head/d d.m.) and feed efficiency (1.53 *vs* 1.39 L/kg d.m.) (P<0.05) during the first fifteen DIM, were higher in treated animals that undergone to retained placenta and metritis compared to sick control ones, as visible in Table 6. If only healthy cows were considered, the difference in milk production was more limited, even if still significant (28.32 L/head/d on average in treated *vs* 27.32 L/head/d in control cows) (P<0.05) (Table 5). Several research shown that retained placenta led to a strong reduction both in feed intake and milk production [29].

No significant effects of the Treatment were seen in terms of monthly milk production and quality during the first three months of lactation, as visible in Table 7 and 8. Also, BCS was not affected by the Treatment, both in terms of average values as well as of distribution in the different classes, as reported in Table 9 and 10.

As a result of a better health status and a faster recover of both calcium and energy balance, also the reproductive parameters were improved by the Treatment (Table 11), with a significantly (P<0.05) lower intervals between calving and first heat (36.55 *vs* 41.86 days) and calving and conception (79.31 *vs* 85.06 days) in Treatment compared to Control group. Also, in sick animals with retained placenta and metritis (Table 12), the intervals between calving and both first heat (53.00 *vs* 67.66 days) and pregnancy (125.50 *vs* 153.67 days) were significantly lower in treated cows compared to controls one (P<0.05). The results highlighted the strong negative effect of both retained placenta

Table 10 - Body condition score distribution in the different classes

BCS thresholds	Treatment	Control	*P*-value
	BCS 5 days before calving, % (n)		
3.25	31.11 (14)	26.67 (4)	ns
3.50	31.11 (14)	26.67 (4)	
3.75	13.33 (8)	33.33 (5)	
4.00	13.33 (8)	13.33 (2)	
4.25	1.67 (1)	0.00 (0)	
	BCS 5 days after calving, % (n)		
2.50	2.22 (1)	0.00 (0)	ns
2.75	33.33 (15)	33.33 (5)	
3.00	35.56 (16)	46.67 (7)	
3.25	24.44 (11)	20.00 (3)	
3.50	4.44 (2)	0.00 (0)	
	BCS at 30 days of lactation, % (n)		
2.75	0.00 (0)	6.67 (1)	ns
3.00	31.11 (14)	26.67 (4)	
3.25	51.11 (23)	40.00 (6)	
3.50	13.33 (6)	26.67 (4)	
3.75	4.44 (2)	0.00 (0)	

Table 11 - Reproductive performance in the two study groups.

Group	Retained placenta, % (n)	Clinical milk fever, % (n)	Metritis, % (n)	Mastitis, % (n)	Lameness, % (n)
Control	20.00 (3)	13.33 (2)	20.00 (3)	0.00 (0)	6.67 (1)
Treatment	8.89 (4)	4.44 (2)	2.22 (1)	2.22 (1)	8.89 (4)
P-value	ns	ns	<0.05	ns	ns

Table 12 - Reproductive performance in cows that undergone to retained placenta and metritis in the two study groups.

Group	Days to first heat, d	Days to first service, d	Days to pregnancy, d	Service to pregnancy, d
		Sick cows (retained placenta, metritis)		
Control	67.66	111.67	153.67	3.00
Treatment	53.00	99.25	125.50	2.25
P-value	<0.05	ns	<0.05	ns
		Healthy cows		
Control	35.41	78.42	95.91	1.83
Treatment	34.95	77.36	94.78	1.80
P-value	ns	ns	ns	ns

and metritis on the reproductive efficiency. Rodriguez et al. (2017), found that normocalcemic cows, were more likely to show their first oestrus sooner after calving than hypocalcaemic cows, even if no correlation was found with the other reproductive parameters [15]. Martinez et al. (2012), reported that both time to first oestrus and pregnancy were delayed in cows with subclinical hypocalcaemia compared with normocalcemic cows [14]. More recently, Caixeta et al. (2017) reported that cows with normocalcaemia were 1.8 times more likely to return in oestrus by the end of the voluntarily waiting period than cows classified as having subclinical hypocalcaemia (defined as blood Ca ≤2.00 mmol/L) [30].

CONCLUSIONS

The results of the present study highlight a potential effectiveness of boluses containing 1,25(OH)$_2$D$_3$ extracted from *S. glaucophyllum* in reducing the incidence and severity of hypocalcaemia in transition dairy cows, with positive effect of both animal welfare and productivity.

The better calcium levels highlighted in the Treatment group around calving, have led to both an important reduction in the incidence and severity of hypocalcaemia-related pathologies, such as retained placenta and metritis, with positive effects on both milk yield and reproductive performances.

References

1. Drackley, J.K. Biology of dairy cows during the transition period: The final frontier? J. Dairy Sci., **1999**, 82(11), pp. 2259-2273.
2. Pascottini, O.B.; Leroy, J.L.M.R.; Opsomer, G. Metabolic Stress in the Transition Period of Dairy Cows: Focusing on the Prepartum Period. Animals, **2020**, 10(8), pp.1419.
3. Venjakob, P.L.; Borchardt, S.; Heuwieser, W. Hypocalcemia-Cow-level prevalence and preventive strategies in German dairy herds. Journal of dairy science, **2017**, 100(11). pp. 9258-9266.
4. Martin-Tereso, J.; Martens, H. Calcium and magnesium physiology and nutrition in relation to the prevention of milk fever and tetany (dietary management of macrominerals in preventing disease). Vet. Clin. North Am. Food Anim. Pract., **2014**, 30, pp. 643-670.
5. Reinhardt, T.A.; Lippolis, J. D.; McCluskey, B.J.; Goff, J.P.; Horst, R.L. Prevalence of subclinical hypocalcemia in dairy herds. Vet. J., **2011**, 188, pp. 122-124.
6. Neves, R.C.; Leno, B.M.; Bach, K.D.; McArt, J. Epidemiology of subclinical hypocalcemia in early-lactation Holstein dairy cows: The temporal associations of plasma calcium concentration in the first 4 days in milk with disease and milk production. Journal of dairy science, **2018**, 101(10), pp.9321-9331.
7. Oetzel, G.R. Effect of calcium chloride gel treatment in dairy cows on incidence of periparturient diseases. J. Am. Vet. Med. Assoc., **1996**, 209, pp. 958-961.
8. McArt, J.A.A.; Neves, R.C. Association of transient, persistent, or delayed subclinical hypocalcemia with early lactation disease, removal, and milk yield in Holstein cows. J. Dairy Sci. **2020**, 103, pp. 690-701.
9. DeGaris, P.J.; Lean, I.J. Milk fever in dairy cows: a review of pathophysiology and control principles. Veterinary journal, **2008**, 176(1), pp. 58-69.
10. Horst, R.; Goff, J.P.; Gill, S.; Dallorso, M.E.; Pawlak, E. Using Solanum Glaucophyllum as a source of 1,25-dihydroxyvitamin D to prevent hypocalcemia in dairy cows. Acta Vet. Scand., **2003**, 44.
11. Wilhelm, A. L.; Maquivar, M.G.; Bas, S.; Brick, T.A.; Weiss, W.P.; Bothe, H.; Velez, J.S.; Schuenemann, G.M. Effect of serum calcium status at calving on survival, health, and performance of postpartum Holstein cows and calves under certified organic management. J. Dairy Sci., **2017**, 100, pp. 3059-3067.
12. Roberts, T.; Chapinal, N.; Leblanc, S.J.; Kelton, D.F.; Dubuc, J.; Duffield, T.F. Metabolic parameters in transition cows as indicators for early-lactation culling risk. J. Dairy Sci., **2012**, 95, pp. 3057-3063.
13. Goff, J.P. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J., **2008**, 176, pp. 50-57.
14. Martinez, N., Risco, C.A., Lima, F.S., Bisinotto, R.S.; Greco, L.F.; Ribeiro, E.S.; Maunsell, F.; Galvao, K.; Santos, J.E. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. J. Dairy Sci., **2012**, 95, pp. 7158-7172;
15. Rodríguez, E. M.; Arís, A.; Bach, A. Associations between subclinical hypocalcemia and postparturient diseases in dairy cows. J. Dairy Sci., **2017**, 100, pp. 7427-7434.
16. Seifi, H.A.; Kia, S. Subclinical hypocalcemia in dairy cows: pathophysiology, consequences and monitoring. Iran. J. Vet. Sci. Technol., **2018**, 9, pp. 1-15.
17. Seifi, H.A.; Kia, S. Subclinical hypocalcemia in dairy cows: pathophysiology, Consequences and monitoring. Iran. J. Vet. Sci. Technol., **2017**, 9, 1-15.
18. Meyer-Binzegger, M.; Ollagnier, C.; Eggerschwiler, L.; Bühler, K.; Schlegel, P.; Meylan, M. Pharmacokinetics of 1,25-dihydroxyvitamin D3 glycosides from Solanum glaucophyllum extract given in a rumen bolus on blood mineral profiles in dry pregnant dairy cows. Research in veterinary science, **2021**, 142, pp. 70-77.
19. Meyer-Binzegger, M.; Ollagnier, C.; Eggerschwiler, L.; Bühler, K.; Meylan, M.; Schlegel, P. Potential of a rumen bolus containing 1,25-dihydroxyvitamin D3 glycosides for the prevention of hypocalcaemia in primiparous and multiparous dairy cows. Animal: an international journal of animal bioscience, **2022**, 16(1).
20. McArt, J.A.A.; Oetzel, G.R. A stochastic estimate of the economic impact of oral calcium supplementation in postparturient dairy cows. J. Dairy Sci., **2015**, 98, pp. 7408-7418.
21. Goff, J.P. Invited review: Mineral absorption mechanisms, mineral interactions that affect acid-base and antioxidant status, and diet considerations to improve mineral status. J. Dairy Sci., **2018**, 101, pp. 1-51.
22. Bachmann, H.; Lanz, M.; Kehrle, S.; Bittner, W.; Toggenburger, A.; Mathis, G.A.; Rambeck, W. Effects of a sustained release formulation of 1,25-dihydroxyvitamin D3-glycosides for milk fever prevention on serum 1,25-dihydroxyvitamin D3, calcium and phosphorus in dairy cows. J. Steroid Biochem. Mol. Biol., **2017**, 173, pp. 301-307.
23. Colotta, F.; Jansson, B.; Bonelli, F. Modulation of inflammatory and immune responses by vitamin D. Journal of Autoimmunity, **2017**, 85, pp. 78-97.
24. National Academies of Sciences, Engineering, and Medicine. Nutrient Requirements of Dairy Cattle: Eighth Revised Edition; The National Academies: Washington, DC, USA, 2021.
25. Edmonson, A.J.; Lean, I.J.; Weaver, L.D.; Farver, T.; Webster, G. A body condition scoring chart for Holstein Dairy Cows. J. Dairy Sci., **1989**, 72, 68-78.
26. Ferguson, J.D.; Galligan, D.T.; Thomsen, N. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci., **1994**, 7, 2695-2703.
27. Murray, R. D.; Horsfield, J.E.; McCormick, W.D.; Williams, H.J.; Ward, D. Historical and current perspectives on the treatment, control and pathogenesis of milk fever in dairy cattle. Vet. Rec., **2008**, 163, pp. 561-565.
28. Youngquist, Robert S., and Walter R. Threlfall. Current therapy in large animal theriogenology. Elsevier Health Sciences, **2006**.
29. Dervishi, E.; Zhang, G.; Hailemariam, D.; Dunn, S.M.; Ametaj, B.N. Occurrence of retained placenta is preceded by an inflammatory state and alterations of energy metabolism in transition dairy cows. J Anim Sci Biotechnol., **2016**, 7:26.
30. Caixeta, L.S.; Ospina, P.A.; Capel, M.B., Nydam, D.V. Association between subclinical hypocalcemia in the first 3 days of lactation and reproductive performance of dairy cows. Theriogenology, **2017**, 94, pp. 1-7.