


# Biocholine in the Diet of Jersey Cows and Its Effects on Rumen Environment, Production Efficiency, and Milk Quality

Michel Breancini<sup>1</sup>, Mateus Henrique Signor<sup>2</sup> , Gabriel Jean Wolschick<sup>1</sup>, Rafael Vinicius Pansera Lago<sup>2</sup>, Natalia Gemelli Corrêa<sup>1</sup>, Maksuel Gatto de Vitt<sup>2</sup>, Karoline Wagner Leal<sup>2</sup>, Andrei L. R. Brunetto<sup>2</sup>, Luiz Eduardo Lobo e Silva<sup>3</sup>, Roger Wagner<sup>3</sup>, Camila T. K. Jung<sup>4</sup>, Gilberto V. Kozloski<sup>4</sup>, Aleksandro Schafer da Silva<sup>1,2\*</sup>

<sup>1</sup>Department of Animal Science, Universidade do Estado de Santa Catarina (UDESC), Chapecó, Brazil

<sup>2</sup>Postgraduate Program in Animal Science, Universidade do Estado de Santa Catarina (UDESC), Chapecó, Brazil

<sup>3</sup>Department of Food Science, Universidade Federal de Santa Maria (UFSM), Santa Maria, Brazil

<sup>4</sup>Department of Animal Science, Universidade Federal de Santa Maria (UFSM), Santa Maria, Brazil

Email: \*aleksandro\_ss@yahoo.com.br

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## Abstract

The study aimed to evaluate whether the use of biocholine (BC) in the diet of dairy cows has positive effects on milk production, reflecting benefits to the cows' health. For the experiment, 14 Jersey cows were used, with an average day in lactation (DL) of 130, with an average weight of 394.5 kg, randomly divided into two groups: the control group (CONT—not-additive) and the treatment group that received inclusion in the concentrate of 10 g BC/cow/day. The experiment lasted 28 days, with blood samples collected for complete blood count and biochemical analysis, milk production and composition, and rumen fluid and feces samples collected to assess digestibility. There was no difference in feed intake during the experimental period, but the animals that received the additive produced 11.52% more milk during the experimental period ( $p \leq 0.05$ ). When milk production was corrected for 4% fat, we found that productivity was 19.1% higher ( $p \leq 0.01$ ) in cows that consumed BC than CONT. A higher apparent digestibility coefficient of crude protein was observed in cows in the treatment group compared to the control ( $p \leq 0.05$ ). The percentage of total solids in the milk of animals in the treatment group was higher ( $p \leq 0.05$ ), concomitant with tendency of lower somatic cell counts during some days of the experimental period ( $p = 0.07$ ). A higher proportion of unsaturated fatty acids in cows fed with BC was observed ( $p \leq 0.05$ ). Acetic, butyric, and valeric acids were in higher percentages, while propionic and iso-valeric were in lower proportions in treatment cows compared to CONT ( $p \leq$

0.05). The activity of hepatic extravasation enzymes (ALT and AST) in serum was lower in the cows that consumed BC ( $p \leq 0.05$ ). The BC intake alters erythrocyte count, hematocrit percentage and hemoglobin concentration on certain days, with higher values in animals in the treatment group compared to the CONT ( $p \leq 0.05$ ). Lower lipid peroxidation and higher activity of anti-oxidant enzymes (glutathione S-transferase and superoxide dismutase) when cows consumed BC compared to the CONT ( $p \leq 0.05$ ). In conclusion, BC intake promotes animal health benefits and contributes to higher milk productivity.

## Keywords

Additive, Hepatoprotective Effect, Nutrient Digestibility, Milk Production

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## 1. Introduction

Performance enhancers in animal production are common in many countries and are no different in dairy farming. A study carried out by Nunes *et al.* [1], with increasing doses of biocholine (BC) for cows in mid-lactation, demonstrated that BC had no effect on nutrient intake but linearly increased fat digestibility without altering the total volatile fatty acids in the rumen; enhancing milk production at a dose of 10 g/animal/day (quadratic effect). Since BC has low ruminal degradation, it is believed to be a source of choline for lactating cows after peak lactation, when the animal leaves the negative energy metabolism and begins to deposit fat; however, there are no publications on the effect of BC on the ruminal microbiota of cows; which would allow a comparison of similar action to monensin as a nutritional growth enhancer; despite the better lipid digestibility [1]. Therefore, BC's primary mechanisms of action reflect improved animal health, consequently influencing productivity [2]-[4].

BC is a source of choline, an alternative to synthetic choline, which is known for its hepatoprotective function, assists lipoproteins in the transport of triglycerides, minimizes oxidative stress, and cannot be converted to trimethylamine [5]. Choline is essential for the synthesis of acetylcholine and phosphatidyl molecules, where acetylcholine is an important neurotransmitter in parasympathetic nervous system [6] and phosphatidyls are essential to maintaining the structure of cellular membrane, synthesis, and secretion of very-low-density lipoprotein in the liver, and synthesis and secretion of milk [1] [6]. BC contains choline as choline conjugates, mainly phosphatidylcholine, a molecule that presents natural resistance against ruminal degradation [7]. Combs Jr. [8] states that choline can be absorbed into cells in three ways: transported via high-affinity sodium-dependent, low-affinity sodium-independent cation transporter, and passive diffusion. In the intestinal lumen, phosphatidylcholine can be acted upon by phospholipases produced by the pancreas (phospholipase that cleaves the two fatty acids to give rise to glyceryl phosphorylcholine); thus, most of the ingested phosphatidylcholine is ab-

sorbed as lyselectin (a product of pancreatic action), as it is recycled in the enterocyte to produce phosphatidylcholine [8].

A meta-analysis also demonstrated that dairy cows benefit from the inclusion of rumen-protected B vitamins and choline during the transition period [9]. Similarly, when researchers added rumen-protected choline to cows' diets during the transition period, there were positive effects on lactation performance and health [10]. Therefore, we hypothesized that BC could act as an enhancer of productive performance in cows, so we chose to compare BC with the main additive used in dairy farming. So, the study aimed to evaluate whether the use of BC in the diet of dairy cows has positive effects on milk production, as well as reflecting benefits to the cows' health.

## 2. Materials and Methods

### 2.1. Animals and Facilities

The experiment was conducted in the Experimental Farm of the *Centro Superior de Educação do Oeste* (FECEO) dairy cattle sector in Guatambu-SC. Latitude (S) G: 27 M: 9 S: 9.52, Longitude (W) G: 52 M: 47 S: 14.22, humid Subtropical climate. The average temperature during the experimental period was 17.7°C, and the relative humidity was 72%, as shown in **Figure S1**, demonstrating that the temperature was within the required parameters, providing thermal comfort conditions. This work was submitted and approved by the Ethics Committee for the use of animals at UDESC under protocol number 8303290323, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA/Brazil).

Fourteen Jersey breed primiparous cows were used, with an average of 130 ± 3.2 DL and an average weight of 394.5 ± 14.7 kg. The cows were under a Compost Barn type of confinement regime with space per animal of 14.4 m<sup>2</sup> on a sawdust bed, availability of MX300 collective tilting drinking fountains (MX do Brasil®) with a capacity of 300 liters, in addition to an Eco fan system. Fan MX 160 (MX do Brasil®) controls the warehouse's internal microclimate. Attached to the shed is the feeding lane, with a drinking fountain milking room used for a milking set of robotic systems (DeLaval VMS™ V300). The milking system was a guided flow type, and the cows had free access to milking only two times during the day, with morning milking starting at 5:00 am and afternoon milking at 3:00 pm.

### 2.2. Additive and Experimental Design

Our study used green powder denominated BC, a commercial product from NutriQuest (Biocholine Powder®); the guaranteed levels expressed in the product description show levels above 32 g/kg of phosphatidylcholine. The additive is produced from the extract of selected plants with high choline content in the esterified form *Azadirachta indica*, *Citrullus colocynthis*, *Trachyspermum ammi*, and *Achyranthes aspera* [11]. This choline source is not hygroscopic, does not convert into trimethylamine, and does not accelerate the destruction of vitamins in the

premix. It also helps to use the choline present in feeds used in the diet. A sample of this additive was used to quantify choline in BC using high-performance thin-layer chromatography, as described by Kupke and Zeugner [12]. The colorimetry method was used to determine tannins, phenolic, and flavonoids. The amount of tannins was measured by colorimetry, following method 52, *Compêndio Brasileiro de Alimentação Animal*, 2013, and of flavonoids was measured following the methodology described by Dazuk *et al.* [13].

The experimental period was 28 days, with 14 initial days of adaptation and 14 days of data and sample collection. The animals with the same sample number were randomly divided into two groups based on body weight and average milk production over the last seven days; in order to have groups with similar characteristics for these variables. One group was defined as control (CONT—not-additive); and another group received the inclusion of BC in their diet at a dose of 10 g per animal per day, as suggested by Nunes *et al.* [1], which was already included in the concentrate during its production.

The feed was provided individually at three different times during the day (6:00, 11:00, and 16:00, with each session lasting 2 hours), entirely delivered at the feeding alley, where the animals were restrained by a headlock and had access to water. The concentrate supplied by the robot during milking, limited to 2 kg per animal per day, was defined as a “milking diet”.

### 2.3. Feed Intake

The total diet was formulated according to the nutritional needs required for each animal [14], formulated based on 395 kg of body weight, for a production of 22 kg/animal/day of milk, DL of 130, using corn silage-based feeds, Tifton 85 hay and corn-based concentrate, cornmeal, soybean bran, wheat bran, soybean hulls, livestock urea, sodium bicarbonate, adsorbent (Milk Sacc®) and mineral core (Bovigold®). Ingredients (corn silage, hay, and concentrate) were mixed and made available to the animals, forming a partial mixed ration (PMR). The cows also received commercial pelleted concentrate (Cooper Alfa) provided during milking; being limited to 2 kg/cow/day (1 kg morning milking; 1 kg afternoon milking).

**Table 1** contains data on the chemical composition of feeds and PMR.

**Table 1.** Chemical composition of silage, basal concentrate, hay, and total diets supplied to cows during the experiment, and fatty acid profile in partial mixed ration (PMR).

Variables. %	Silage	Pelleted concentrate	Basal concentrate	Hay	PMR control group	PMR treatment group
Dry matter	25.09	88.2	88.75	87.65	39.95	40.05
Crude protein	9.88	18	22.52	13.44	16.03	16.09
Etherial extract	2.90	4.36	3.53	1.63	2.98	3.02
Ash	3.74	6.82	10.79	6.73	7.13	6.94

## Continued

ADF	19.62	9.1	9.37	33.67	17.27	18.04
NDF	40.35	19.96	21.46	69.63	38.82	35.73
C10:0 (Capric)	-	-	-	-	0.097	0.054
C12:0 (Lauric)	-	-	-	-	0.287	0.300
C14:0 (Myristic)	-	-	-	-	0.867	0.561
C15:0 (Pentadecanoic)	-	-	-	-	0.182	0.125
C16:0 (Palmitic)	-	-	-	-	22.73	21.11
C16:1 (Palmitoleic)	-	-	-	-	0.707	0.482
C17:0 (Heptadecanoic)	-	-	-	-	0.599	0.490
C18:0 (Stearic)	-	-	-	-	6.571	4.518
C18:1n9t (Elaidic)	-	-	-	-	0.397	0.173
C18:1n9c (Oleic)	-	-	-	-	25.15	24.35
C18:2n6c (Linoleic)	-	-	-	-	33.70	38.51
C20:0 (Arachidic)	-	-	-	-	0.763	0.765
C20:1n9 (cis-11-Eicosenoic)	-	-	-	-	0.346	0.346
C18:3n3 (a-Linolenic)	-	-	-	-	5.217	5.721
C22:0 (Behenic)	-	-	-	-	0.796	0.911
C22:1n9 (Erucic)	-	-	-	-	0.249	0.236
C20:4n6 (Arachidonic)	-	-	-	-	0.284	0.105
C24:0 (Lignoceric)	-	-	-	-	0.876	1.028
C24:1n9 (Nervonic)	-	-	-	-	0.168	0.200
Σ Saturated fatty acids (SFA)	-	-	-	-	33.77	29.86
Σ Unsaturated fatty acids (UFA)	-	-	-	-	66.22	70.13
Σ Monounsaturated fatty acids	-	-	-	-	27.02	25.78
Σ Polyunsaturated fatty acids	-	-	-	-	39.20	44.34
UFA/SFA	-	-	-	-	1.961	2.349
Σ ω6	-	-	-	-	33.98	38.62
Σ ω3	-	-	-	-	5.217	5.721
ω6/ω3	-	-	-	-	6.518	6.758

Note 1: The ingredients present in the concentrate were: ground corn (51.8%), soybean meal (32.14%), soybean hulls (5.24%), wheat bran (4.76%), urea (0.6%), sodium bicarbonate (1.67%), adsorbent (0.8%) and mineral core (3%). The guarantee levels of the mineral and vitamin core are Ca min. 231.40 max. 250 g; P min 40; S min 20 g; Mg min. 25 g; K min. 10 g; At min. 70 g; with min. 15 mg; Ass. min. 15 mg; Cr min. 20 mg; I. min. 40 mg; Mg. Min 2000 mg; min. 22 mg; Zn. Min 2850 mg. Vit A. 350,000 IU; Vit D3 min. 100,000 IU; Vit. E. 2000 IU.

## 2.4. Sample and Data Collection

Blood samples were collected on days 1, 14, 21, and 28 for blood count and metabolic biochemistry using needles and vacuolated tubes; tubes without anticoagulants were used for biochemical analyses and with anticoagulant (EDTA) for hematologic analyses. The tubes were kept refrigerated at 10°C until arrival at the laboratory (box with styrofoam and ice). Subsequently, the blood was centrifuged at 7000 rpm for 10 minutes to separate the serum. Then, a serum fraction was stored in 1.8-ml microtubes (Eppendorf) and frozen (−15°C) for subsequent analysis. During random moments of the experiment, hay, silage, concentrate, and total PMR were collected from the control and treatment groups, and the material was used to form a pool that represents the experimental period for subsequent chemical analysis of the feed.

Individual milk analyses were collected from each animal on days 1, 14, 21, 25, and 28, using the automatic collection system (DeLaval VMS™ V300), stored at 10°C in styrofoam boxes with ice until arrival at the laboratory, and subsequently, analyses of the proximate composition of the milk were carried out; the remainder was frozen in Eppendorf for analysis of the milk's fatty acid profile.

In the robotic milking system (DeLaval VMS™ V300), individual production data for each animal were obtained during the experimental period, and data on Somatic cell counts (SCC), body condition score (BCS), and individual pelleted concentrate intake. Milk production corrected for 4% fat (4% FCM) was estimated by the equation proposed by the NRC [14]:  $FCM = 0.4 \times (\text{kg of milk produced}) + 0.15 \times (\% \text{ fat}) \times (\text{kg of milk produced})$ .

On the last day of the experiment, rumen fluid was collected using a silicone esophageal probe attached to a vacuum system. The pH of the collected material was measured within 1 minute after collection, and the material was then filtered using double gases. The rumen fluid was stored in microtubes and frozen at −80°C until analysis.

## 2.5. Laboratory Analysis

### 2.5.1. Analysis of the Chemical Composition of Feed, Biocholine, and Feces

To conduct chemical composition analyses of the feeds used, homogeneous samples of the total diet of both experimental groups were collected. These samples were pre-dried in a forced air oven at 55°C for 72 hours, removed from the oven, and weighed to determine the partial DM content, followed by grinding in a Wiley-type mill (Marconi, model: MA340), using a sieve of 1-mm mesh. The pre-dried and ground samples were heated at 105°C to obtain the DM and the mineral material in a muffle furnace at 600°C [15]. Crude protein (CP) was determined using the micro-Kjeldahl method (Method 984.13, AOAC, 1997). To determine the neutral detergent fiber (NDF) content, the samples were placed in polyester bags (Komarek, 1993) and treated with a neutral detergent solution in an ANKOM 200® fiber analyzer at 100°C for 1 hour. Acid detergent fiber (ADF) concentrations

were determined according to AOAC (1997, method 973.18). The ether extract portion of the samples was quantified using an automatic SER158 Fat Extractor (VELP® Scientifica).

Ten fecal samples were collected during the experimental period of three days (d 22, 23, 24, 25, and 26), with the material collected directly from the cow's rectal ampulla. The material was frozen, and at the end of the experiment, a pool of all collections in that period was formed with 500 g. The material was dried in a forced circulation oven at 56°C for 72 h. It was then ground in a knife mill, removed from the oven, weighed to determine the partial DM content, and ground in a Wiley-type mill (Marconi, model: MA340) using a 2 mm mesh sieve. The pre-dried and ground samples were heated at 105°C to obtain the MS and the mineral material in a muffle furnace at 600°C [15].

Samples were analyzed according to AOAC [16]: DM, method 930.15; CP, method 976.05; ether extract (EE), method 920.39 and ash, method 942.05. The concentrations of NDF and ADF were determined according to Van Soest *et al.* [17] methodology without adding sodium sulfite or alpha-amylase.

### 2.5.2. Apparent Digestibility

We used indigestible neutral detergent fiber (iNDF) to determine apparent digestibility, as described by researchers [18]. The feed and feces samples were incubated in bovine rumen for 288 hours, washed, and dried in a forced ventilation oven. NDF and ADF concentrations were determined to calculate digestibility [19].

### 2.5.3. Hemogram

The hematologic variables were obtained using tubes of blood with EDTA collected, using a VET3000 hematologic analyzer (equip®), which determines the following variables: leukocytes, lymphocytes, granulocytes, monocytes, total erythrocytes, and platelet count in cells  $\times 10^3/\mu\text{l}$ , total red blood cell count ( $\times 10^6/\mu\text{l}$ ), hemoglobin concentration (g/dL) and hematocrit (%).

### 2.5.4. Serum Biochemistries

Subsequently, the serum was stored in microtubes at  $-20^\circ\text{C}$  until analysis. Total protein (PT), albumin (AL), glucose, urea, triglycerides, and total cholesterol levels were evaluated in serum. The ANALISA® kits were used in semi-automatic equipment (Bio Plus 2000®). The concentration of globulins will be calculated using the PT-AL equation.

Lipoperoxidation is a highly rapid reaction formed by the breakdown of polyunsaturated fatty acids, which are usually measured by their products, mainly TBARS, among which malondialdehyde (MDA) is the primary one [20]. To evaluate this product, the reaction of thiobarbituric acid (TBA) with serum samples was used, which, in the presence of MDA, results in a pink product that can be read at 532 nm. Briefly, 20  $\mu\text{L}$  of samples were mixed with 55  $\mu\text{L}$  of distilled water, 100  $\mu\text{L}$  of orthophosphoric acid (0.2 M), and 25  $\mu\text{L}$  of TBA (0.1 M). A spectrophotometric reading was taken after 45 minutes of incubation at 37°C. Results were

expressed in nM MDA/mL. Serum glutathione S-transferase (GST) activity was measured based on the method described by Habig *et al.* [21] and expressed as U GST/mg protein. The measurement of SOD activity was based on the inhibition of the radical superoxide reaction with adrenaline, as described by McCord and Fridovich [22]. In this method, SOD present in the sample competes with the detection system for radical superoxide. One SOD unit is defined as the amount of enzyme able to slow down the speed of adrenaline oxidation by 50%. The oxidation of adrenaline leads to the formation of colored product adrenochrome that might be detected by spectrophotometry. SOD activity was determined by measuring the speed of adrenochrome formation observed at 480 nm in a reaction medium containing glycine-NaOH (50 mM, pH 10.0) and 1 mM adrenaline. Specific activity was expressed as SOD units per mg of protein.

### 2.5.5. Beta-Hydroxybutyrate Measurement

For the measurements of ketosis in animals, blood assessment of beta-hydroxybutyrate was carried out using a device from the Free Style Optium Neo human line, which determines the concentration of ketone bodies through a drop of blood taken from the cocchial vein and placed on the tape of the device. The reference values for BHB, according to Duffield *et al.* [23], BHB  $\leq$  1.1 mmol/L—reflects the absence of ketosis; BHB  $\geq$  1.2 mmol/L subclinical ketosis and BHB; 3.5 mmol/L clinical ketosis.

### 2.5.6. Profile of Short-Chain Fatty Acids in Rumen Fluid

Ruminal fluid samples were thawed at 5°C and manually shaken for homogenization. Aliquots of 1 mL of the supernatant of ruminal fluid samples were collected in polypropylene microtubes (2 mL) and then centrifuged for 5 minutes ( $12,300 \times g$ ). Then, 250  $\mu$ L of the supernatant was transferred to a new microtube containing 250  $\mu$ L of formic acid. The mixture was manually shaken and centrifuged for 3 min. After centrifugation, 250  $\mu$ L of the supernatant of the mixture was collected in another polypropylene tube previously containing 500  $\mu$ L of isoamyl alcohol (3-methyl-1-butanol) solution (638.28  $\mu$ g·mL<sup>-1</sup> in methanol), used as an internal standard, and were homogenized and centrifuged again. 650  $\mu$ L of sample was inserted into a 2-mL injection vial; 1  $\mu$ L of extract was injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3400, Palo Alto, USA) and an autosampler (Varian 8200CX, Palo Alto, USA) in split mode (1:10) at 250°C. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric, and isovaleric acids) were separated by a CP-Wax 52CB capillary column (60 m  $\times$  0.25 mm; 0.25  $\mu$ m stationary phase thickness). The initial column temperature was set at 80°C for 1 min and increased to 120°C at a rate of 8°C min<sup>-1</sup>, then to 230°C for 20°C min<sup>-1</sup>, where it remained for 1 min. The detector temperature was set to 250°C. Method validation comprised the following parameters: selectivity, linearity, linear range, repeatability, precision, limit of detection (LOD), and limit of quantification (LOQ) for acetic, propionic, butyric, and isovaleric acids. Linearity was assessed

by calculating a regression equation using the least squares method. LOD and LOQ values were obtained by sequential dilutions to signal-to-noise ratios of 3:1 and 6:1, respectively. Precision was assessed by analysing the repeatability of six replicated samples. Accuracy was determined by recovering known amounts of the standard substances added to a diluted sample (Table S1). Valeric acid was expressed as the equivalent of isovaleric acid. The results were expressed in mol 100 mol<sup>-1</sup> of each short-chain fatty acid (SCFA) in the ruminal fluid.

### 2.5.7. Milk Composition and Fatty Acid Profile

In the laboratory immediately after collection, the milk samples were analyzed using infrared equipment (LactoStar Funke Gerber®), quantifying the proximate composition of fat, protein, lactose, and total milk solids.

Lipid extraction was performed using the method of Bligh and Dyer [24] with adaptations to determine the fatty acid profile in milk. 1.5 g of samples, 0.8 mL of water, 5 mL of methanol, and 2.5 mL of chloroform were added to a 15 mL polypropylene tube, and mechanical stirring was carried out for 30 min. Next, 2.5 mL of chloroform solution and 1.5% NaSO<sub>4</sub> were added to promote a two-phase system. This mixture was stirred for 2 minutes and then centrifuged for 15 minutes at 2000 rpm. The lipids obtained from the chloroform phase were subjected to fatty acid analysis.

FA methylation was performed using a transesterification method proposed by Hartman and Lago [25]. 1 mL of 0.4 M KOH methanolic solution was added to the extracted lipids in a test tube and vortexed for 1 min. The samples were kept in a water bath for 10 minutes at boiling temperature. Subsequently, they were cooled to room temperature, and 3 mL of 1 M methanolic H<sub>2</sub>SO<sub>4</sub> solution were added, vortexed, and kept in a water bath for 10 min. After cooling, 2 mL of hexane was added and centrifuged at 2000 rpm for 10 min. Finally, hexane with fatty acid methyl esters (FAME) was subjected to chromatographic analysis.

A gas chromatograph model TRACE 1310 with a flame ionization detector (Thermo Scientific) was used to determine FAME. One microliter of samples was injected into a split/splitless injector, operated in split mode with a 1:20 ratio at 250°C. Hydrogen was used as the carrier gas at a constant flow rate of 1.5 mL/min. Separation of FAMEs was performed using an RT 2560 chromatography column (100 m × 0.25 mm × 0.20 µm film thickness, Restek, USA). The initial oven temperature was programmed at 100°C for 5 min and increased to 180°C at an 8°C/min rate. Then, it increases to 210°C at a rate of 4°C/min, and finally up to 250°C, increases to 20°C/min, and maintains for 7 min in isothermal. The detector temperature was kept constant at 250°C. FAME compounds were identified by comparing the experimental retention time with those of the authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO). The results were presented as a percentage of each FA identified in the lipid fraction, considering the factor equivalent to the size of the FAME chain for FID and the ester conversion factor for the respective acid, according to Visentainer and Franco [26].

## 2.6. Statistical Analysis

All variables were subjected to the Shapiro-Wilk W-test, which revealed a normal data distribution. Skewness, kurtosis, and homogeneity were evaluated using the Levene test, and linearity was used using linear regression. All data were analyzed using the SAS MIXED procedure (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed effects (day, treatment, and day  $\times$  day interaction) test in a completely randomized design. Fat-corrected milk 4%, dairy efficiency, feed conversion, sum of milk production, and average production gain were tested for fixed treatment effects using animal (within each group) as random variable. All other variables (milk production, composition, quality, serum biochemistry, blood count) were analyzed as repeated measurements and tested for fixed effects of treatment, day, and treatment  $\times$  day interaction (group) and animal as random variables. All results obtained in d1 for each variable were also included as covariates; however, the command for covariates was removed from the model when  $p > 0.05$ . The mean comparison analysis was performed based on PDIFF, the Student's test was chosen due to the experimental condition of only two groups. All results were reported as LSMEANS followed by standard error. Significance was defined when  $p \leq 0.05$ .

## 3. Results

### 3.1. Biocholine (BC)

The product has the following chemical composition: 978 g/kg of DM; 885 g/kg of organic material; 40 g/kg protein; 456 g/kg NDF; 367 g/kg ADF; and 52 g/kg EE. Analysis of the BC allowed identifying 88.5% of the composition of the commercial product, of which 56% was total choline (10.6% phosphatidylethanolamine, 16.8% phosphatidylinositol, 55.2% phosphatidylcholine, 9.59% lyso phosphatidylcholine, and 7.81% phosphatidylcholine “natural choline conjugates”), 8.72 g/kg total tannins, 2.53 g/kg total flavonoids.

### 3.2. Body Weight and Body Condition Score

Body weight and BCS results are shown in **Figure S2**. BW and BCS of the evaluated animals had no significant difference between the groups.

### 3.3. Productive Performance

Productive performance results are presented in **Table 2**. Feed intake showed no statistical difference when considering the adaptation and experimental periods (**Table 2**;  $p > 0.05$ ). Milk production during the experiment period was higher in BC cows compared to the control group, with an interaction between treatments  $\times$  day ( $p \leq 0.05$ ), with this production being 11.55% higher compared to the control group of animals ( $p \leq 0.05$ ). The average milk production kg/day tended to be higher in relation to the control group of animals, and the sum of milk produced per cow during d15-28, throughout the experimental period, was more significant

than the control group of animals. Considering the period from 1 to 28 days, we found that there was a treatment x day interaction for milk production (**Figure 1**;  $p \leq 0.05$ ); being greater in animals that consumed BC on some days. Milk production corrected to 4% fat was 19.1% higher in cows that consumed BC compared to CONT ( $p \leq 0.05$ ) in the experimental period (d15 to 28). No effect of the treatment was found on productive efficiency and feed conversion ( $p > 0.05$ ).

**Table 2.** Productive performance and milk composition of Jersey cows fed with biocholine (treatment group) compared to control group.

Variables	Control	Treatment	SEM	p: treat	p: treat × day
<b>Feed intake/day (kg MS)</b>					
Adaptation period (d1 to 14)	14.9	15.7	0.36	0.81	0.74
Experiment period (d15 to 28)	13.2	14.7	0.41	0.32	0.19
<b>Average milk production (kg/day)</b>					
Adaptation period (d1 to 14)	19.1	19.9	0.38	0.92	0.91
Experimental period (d15 to 28)	17.6 <sup>b</sup>	19.9 <sup>a</sup>	0.35	<b>0.05</b>	<b>0.02</b>
<b>Fat-corrected milk (4%FCM)<sup>3</sup></b>					
Experimental period (d15 to 28)	18.7	22.3	0.41	<b>0.01</b>	-
<b>Sum of milk produced/cow</b>					
(d1-14)	268	279	4.09	0.56	-
(d15-28)	247 <sup>b</sup>	279 <sup>a</sup>	5.38	<b>0.05</b>	-
(d1-28)	516 <sup>b</sup>	558 <sup>a</sup>	8.36	<b>0.02</b>	-
Average gain in milk produced kg/day (d1-14 to d15-28)	-1.50 <sup>b</sup>	0.04 <sup>a</sup>	0.05	<b>0.09</b>	-
<b>Dairy efficiency. kg/kg</b>					
Adaptation period (d1 to 14)	1.28	1.27	0.03	0.92	-
Experimental period (d15 to 28)	1.36	1.35	0.02	0.94	-
<b>Feed conversion. kg/kg</b>					
Adaptation period (d1 to 14)	0.78	0.79	0.01	0.93	-
Experimental period (d15 to 28)	0.74	0.74	0.01	0.94	-
<b>Milk composition</b>					
Fat (g/100g)	4.45 <sup>b</sup>	4.83 <sup>a</sup>	0.11	0.10	0.05
Protein (g/100g)	3.47 <sup>b</sup>	3.78 <sup>a</sup>	0.08	0.05	0.01
Lactose (g/100g)	4.88	5.20	0.08	0.05	0.01
Total solids (g/100g)	12.8	13.8	0.10	0.05	0.02

Note 1: Means followed by letters written differently (a, b) on the same line illustrate a treatment effect when considering  $p \leq 0.05$  and a tendency to be different when  $p > 0.05$  and  $\leq 0.10$ ; Note 2: Details of the treatment × day interaction were presented in **Figure 1** and Supplementary material 4; <sup>3</sup>Milk production corrected for 4% fat (4% FCM) was estimated by the equation proposed by the NRC (2001):  $FCM = 0.4 \times (\text{kg of milk produced}) + 0.15 \times (\% \text{ fat}) \times (\text{kg of milk produced})$ .

### 3.4. Centesimal Composition of Milk

The proximate milk composition results are presented in **Table 2** and **Table S2**. The results show a treatment effect for the percentage of protein, lactose, and total solids, which is more significant in cows that consumed BC than in the control group ( $p \leq 0.05$ ). This difference is related to the interaction of treatment  $\times$  days on d14, d21, and d28 ( $p \leq 0.05$ ). The variables protein, lactose, and total solids were higher in the milk cows in the treatment group ( $p \leq 0.05$ ). The percentage of fat tended to be different, with the fat in the milk produced by animals in the treatment group being higher compared to the percentage of fat in the control animals, with a statistical difference in the interaction of treatment  $\times$  days on d14 and d28, higher in the group of animal treatment ( $p \leq 0.05$ ).

There was a day effect for fat, protein, and total solids in milk, data presented. Over time, fat levels increased in the animals in the treatment group ( $p \leq 0.05$ ); this did not happen in the control group. Protein in milk was lower on day 14 compared to other collection days in the cows in the control group ( $p \leq 0.05$ ). Total solids in milk differed in both groups, but the highlight was in the treatment group, which increased over time.

### 3.5. Milk Fatty Acid Profile

The results of the milk fatty acid profile are described in **Table 3**. The percentage of C15:0 and C21:0 fatty acids was lower in the milk of cows in the treatment group, with an interaction between treatment versus day on d14 and d28 ( $p \leq 0.05$ ). The effect of treatment for C17:0 was observed, with milk from cows in the treatment group being lower. The percentage of C18:0 tended to the interaction between treatment  $\times$  day, being higher in the milk produced by the treatment group of animals than in the control group (d-28). The treatment for C18:1n9c had an effect that was more significant in cows fed with BC ( $p \leq 0.05$ ). Treatment  $\times$  day interaction for C18:2n6c was observed, being greater in cows that consumed additive on d14 and d28. The percentage of C18:3n6 was higher in the milk of treatment cows compared to the control group of animals ( $p \leq 0.05$ ). The sum of unsaturated fatty acids was higher in the milk of cows in the treatment group compared to the control group ( $p \leq 0.05$ ). There was significant interaction between treatment  $\times$  day on d14 and d28 ( $p \leq 0.05$ ), highlighting this more significant amount of unsaturated fatty acids. Profile of other fatty acids showed no statistical difference ( $p > 0.05$ ).

**Table 3.** Profile of fatty acids in the milk of Jersey cows fed with biocholine (treatment group) and control group.

	Control	Treatment	SEM	p: Treat	p: day	p: treat $\times$ day
C6:0 (Caproic)	0.59	0.63	0.03	0.72	0.52	0.66
C8:0 (Caprylic)	0.55	0.60	0.02	0.31	0.20	0.25
C10:0 (Capric)	2.09	2.14	0.08	0.75	0.32	0.46

## Continued

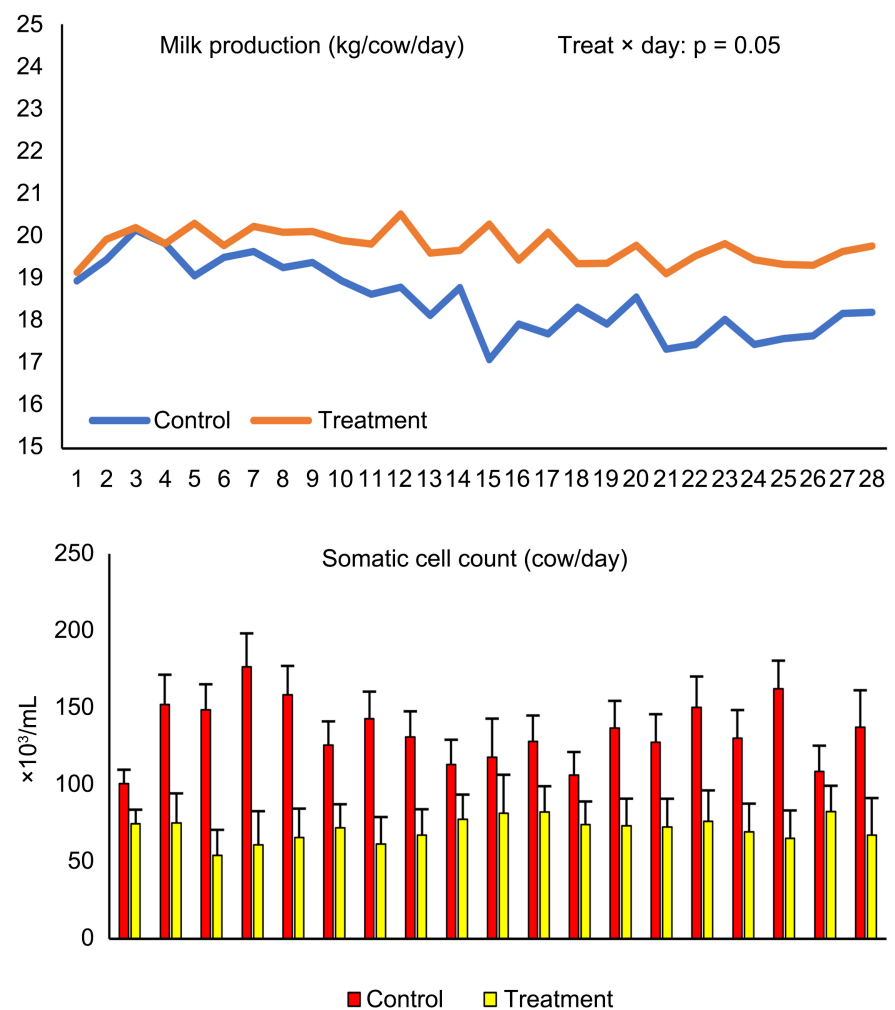
C11:0 (Undecanoic)	0.24	0.23	0.02	0.94	0.91	0.92
C12:0 (Lauric)	3.33	3.33	0.05	0.97	0.95	0.96
C13:0 (Tridecanoic)	0.20	0.17	0.03	0.85	0.72	0.80
C14:0 (Myristic)	11.8	11.5	0.08	0.89	0.88	0.83
C14:1 (Myristoleic)	0.91	0.88	0.06	0.90	0.95	0.92
C15:0 (Pentadecanoic)	1.52 <sup>a</sup>	1.21 <sup>b</sup>	0.03	0.05	0.05	0.01
C16:0 (Palmitic)	44.2	45.0	0.25	0.86	0.79	0.71
C16:1 (Palmitoleic)	1.51	1.40	0.03	0.24	0.21	0.16
C17:0 (Heptadecanoic)	0.71 <sup>a</sup>	0.59 <sup>b</sup>	0.01	0.05	0.11	0.13
C17:1 (cis-10-Heptadecenoic)	0.22	0.18	0.05	0.32	0.26	0.19
C18:0 (Stearic)	9.80	10.3	0.30	0.22	0.22	0.10
C18:1n9c (Oleic)	16.4 <sup>b</sup>	17.4 <sup>a</sup>	0.14	0.05	0.16	0.18
C18:2n6c (Linoleic)	1.96 <sup>b</sup>	3.48 <sup>a</sup>	0.05	0.01	0.01	0.01
C20:0 (Arachidic)	0.17	0.11	0.02	0.21	0.22	0.15
C18:3n6 (?-Linolenic)	0.02 <sup>b</sup>	0.10 <sup>a</sup>	0.01	0.05	0.14	0.11
C20:1n9 (cis-11-Eicosenoic)	0.04	0.03	0.00	0.94	0.98	0.96
C18:3n3 (a-Linolenic)	0.15	0.17	0.01	0.88	0.87	0.9
C21:0 (Henicosoic)	0.43 <sup>a</sup>	0.33 <sup>b</sup>	0.02	0.08	0.21	0.23
C20:2 (cis-11.14-Eicosadienoic)	0.02	0.03	0.00	0.98	0.96	0.97
C22:0 (Behenic)	0.09	0.09	0.00	0.95	0.98	0.98
C20:3n6 (cis-8.11.14-Eicosatrienoic)	0.08	0.10	0.01	0.77	0.95	0.84
C20:4n6 (Arachidonic)	0.03	0.04	0.00	0.97	0.97	0.96
C24:0 (Lignoceric)	0.05	0.06	0.00	0.92	0.93	0.97
C24:1n9 (Nervonic)	0.01	0.01	0.00	0.99	0.99	0.99
Σ Saturated fatty acids (SFA)	75.3	76.1	0.26	0.38	0.25	0.22
Σ Unsaturated fatty acids (UFA)	21.4 <sup>b</sup>	23.8 <sup>a</sup>	0.11	0.02	0.02	0.01
Σ Monounsaturated fatty acids (MUFA)	19.0	19.9	0.11	0.82	0.65	0.74
Σ Polyunsaturated fatty acids (PUFA)	2.30	2.36	0.03	0.89	0.95	0.91
UFA/SFA	0.28	0.28	0.01	0.98	0.98	0.97
Σ ω6	2.11	2.15	0.02	0.92	0.89	0.86
Σ ω3	0.17	0.18	0.01	0.95	0.94	0.95
ω6/ω3	12.6	11.9	0.09	0.24	0.11	0.17

Note 1: Means followed by letters written differently (a, b) on the same line illustrate a treatment effect when considering  $p \leq 0.05$  and a tendency to be different when  $p > 0.05$  and  $\leq 0.10$ ; Note 2: Details of the treatment  $\times$  day interaction and day effect were presented in Supplementary Material 4.

A day effect for C15:0 (pentadecanoic), which in the milk of control cows increased on days 14 and 28 compared to d1 ( $p \leq 0.05$ ), while C18:2n6c (linoleic) also increased in cows that consumed BC on days 14 and 28 compared to d1 ( $p \leq 0.05$ ). Unsaturated fatty acids had a day effect in both groups, but with opposite effects; *i.e.*, in the milk of control cows, UFA levels decreased, while in the treatment cows, they increased ( $p \leq 0.05$ ; **Table S2**).

### 3.6. SCC

SCC results are presented in **Figure 1**. There was no difference between groups for SCC during the experimental period ( $p > 0.05$ ). However, there was a tendency ( $p = 0.07$ ) in the interaction between treatments  $\times$  day, being lower in animals in the group that consumed BC compared to the control group on some days of evaluation. Numerically, the cell count (SCC) was lower in animals that consumed BC throughout the experiment ( $p > 0.05$ ) (**Figure 1**).



**Figure 1.** Milk production (kg/cow/day), and SCC of Jersey cows fed with biocholine (treatment group) and control group (no-additive). The asterisk (\*) illustrates the days where there is a statistical difference between groups.

### 3.7. Hematologic Analysis

Blood count results are presented in **Table 4** and **Table S3**. There was a treatment effect on erythrocyte count and hemoglobin concentration, being more significant in the treatment group compared to the control group ( $p \leq 0.05$ ), with an interaction between treatment  $\times$  day in the experimental period (d14, d21, and d28). Hematocrit showed a statistical difference between the treatment and the control, higher in cows that consumed BC due to the interaction between treatment  $\times$  day (d14 and d28). The number of platelets in animals in the treatment group was lower compared to the control group, with an interaction between treatment  $\times$  day on d21 and d28 ( $p \leq 0.05$ ). There was no statistical difference for the other variables analyzed in the blood count: leukocytes, lymphocytes, granulocytes, and monocytes ( $p > 0.05$ ).

A day effect on hemoglobin concentration increased in animals in the treatment group ( $p \leq 0.05$ ). Platelet count had a day effect in both groups, with an increase in control cows and a decrease in treatment cows ( $p \leq 0.05$ ); however, in both situations, they remained within the reference values for the breed. No effect of day for erythrocyte, leukocyte, granulocyte, neutrophil and monocyte counts; as well as hematocrit percentage ( $p > 0.05$ ).

### 3.8. Biochemistries

**Table 4** and **Table S3** describe the serum biochemistry and metabolism analyses during the experimental period. It is noted that there was an effect of treatment on the enzymatic activity of ALT and AST; that is, it was lower in the serum of animals in the treated group ( $p \leq 0.05$ ), and there was an interaction between treatment  $\times$  day (d21 and d28). There was an interaction between treatment  $\times$  days for the uric acid variable on d14 and d21, which was higher in the treatment group compared to the control group. Glucose levels differed in the control group, being smaller and interacting between treatment  $\times$  day ( $p \leq 0.05$ ; d21). The variables cholesterol, urea, total protein, albumin, globulin, GGT enzyme, and BHB did not show statistical differences during the experimental period ( $p > 0.05$ ).

The effect of day for AST and ALT in cows in the control group was that the activity of these enzymes increased, which did not occur in the treatment group ( $p \leq 0.05$ ). Uric acid levels had an effect of day, being higher on days 14 and 21 when compared to d 1 and 28 in cows that consumed BC ( $p \leq 0.05$ ). There was also an effect of day on glucose; in both groups, it increased throughout the experiment ( $p \leq 0.05$ ).

TBARS levels were lower in cows that consumed BC than in the control ( $p \leq 0.05$ ); however, there was no effect of day or treatment  $\times$  day interaction. GST activity impacted day, treatment, and interaction, emphasizing more significant enzyme activity in the serum of animals that consumed BC ( $p \leq 0.05$ ). SOD activity had an effect of day and treatment  $\times$  day interaction, being higher on day 28 in the blood of cows in the treatment group ( $p \leq 0.05$ ).

**Table 4.** Blood count, serum biochemistry, and oxidative status of cows fed with biocholine (treatment group) and control group.

Variables	Control	Treatment	SEM	p: treat	p: day	p: treat x day
Erythrocytes ( $\times 10^6/\mu\text{L}$ )	5.43 <sup>b</sup>	6.00 <sup>a</sup>	0.07	0.04	0.17	0.01
Hemoglobin (mg/dL)	9.44 <sup>b</sup>	10.8 <sup>a</sup>	0.10	0.02	0.01	0.01
Hematocrit (%)	26.0 <sup>b</sup>	27.5 <sup>a</sup>	0.24	0.05	0.83	0.02
Platelets ( $\times 10^3/\mu\text{L}$ )	313 <sup>b</sup>	236 <sup>a</sup>	22.6	0.01	0.01	0.01
Leukocytes ( $\times 10^3/\mu\text{L}$ )	5.83	6.23	0.19	0.45	0.65	0.68
Lymphocytes ( $\times 10^3/\mu\text{L}$ )	3.24	3.49	0.12	0.52	0.38	0.30
Granulocytes ( $\times 10^3/\mu\text{L}$ )	1.53	1.59	0.09	0.78	0.51	0.63
Monocytes ( $\times 10^3/\mu\text{L}$ )	1.06	1.15	0.05	0.36	0.29	0.22
ALT (U/L)	32.8 <sup>a</sup>	28.7 <sup>b</sup>	0.68	<b>0.03</b>	0.01	<b>0.01</b>
AST (U/L)	130 <sup>a</sup>	111 <sup>b</sup>	3.85	<b>0.01</b>	<b>0.01</b>	<b>0.01</b>
GGT (U/L)	44.5	47.3	3.12	0.59	0.37	0.43
Uric acid (mg/dL)	0.87	0.96	0.05	0.40	<b>0.04</b>	<b>0.05</b>
Glucose (mg/dL)	63.9	58.6	1.18	<b>0.05</b>	<b>0.01</b>	<b>0.05</b>
Cholesterol (mg/dL)	141	137	4.52	0.92	0.77	0.87
Urea (mg/dL)	54.6	54.1	1.12	0.95	0.94	0.92
Total protein (g/dL)	7.50	7.24	0.12	0.62	0.58	0.51
Albumin (g/dL)	3.20	3.18	0.06	0.96	0.90	0.94
Globulin (g/dL)	4.30	4.06	0.08	0.34	0.25	0.18
Beta-hydroxide butyrate (mmol/L)	1.00	1.10	0.02	0.95	-	-
TBARS (nmol MDA/mL)	14.4 <sup>a</sup>	9.04 <sup>b</sup>	1.41	0.05	0.15	0.12
GST (U GST/mg protein)	233 <sup>b</sup>	252 <sup>a</sup>	3.47	0.01	0.01	<b>0.01</b>
SOD (U SOD/mg protein)	5.76	6.53	0.35	0.20	0.01	<b>0.01</b>

Note 1: Means followed by letters written differently (a, b) on the same line illustrate a treatment effect when considering  $p \leq 0.05$  and a tendency to be different when  $p > 0.05$  and  $\leq 0.10$ ; Note 2: Details of the treatment  $\times$  day interaction and day effect were presented in Supplementary Material 5.

### 3.9. Profile of SCFA in Rumen Fluid

The results of the SCFA profile of the rumen fluid are described in **Table 5** and **Table S4**. There was no effect of treatment and no treatment  $\times$  day interaction for SCFA ( $p > 0.05$ ). However, treatment and interaction affected the proportion of acetic, propionic, butyric, isovaleric, and valeric fatty acids ( $p \leq 0.05$ ). The percentage of acetic and butyric acid was higher in animals in the treatment group on D14 and D28 ( $p \leq 0.05$ ) compared to the control. Propionic and isovaleric acid were in lower proportions in cows in the treatment group compared to the control

on days 14 and 28 ( $p \leq 0.05$ ). There was a treatment effect for the proportion of valeric acid, which was more significant in the animals fed with BC ( $p \leq 0.05$ ). There was no effect of day on variables of the SCFA profile in the rumen ( $p > 0.05$ ).

**Table 5.** Profile of short-chain fatty acids and apparent digestibility coefficient (ADC) of nutrients in the cows that consumed biocholine.

Variables	Control	Treatment	SEM	p: treat	p: day	p: day × treat
<b>Fatty acid</b>						
SCFA (mmol/L)	108	110	0.94	0.92	0.90	0.88
Acetic acid, %	64.0 <sup>b</sup>	66.5 <sup>a</sup>	0.56	0.05	0.95	0.02
Propionic acid, %	22.5 <sup>a</sup>	18.5 <sup>b</sup>	0.20	0.01	0.97	0.01
Butyric acid, %	10.4 <sup>b</sup>	12.6 <sup>a</sup>	0.15	0.03	0.93	0.01
Isovaleric acid, %	1.83 <sup>a</sup>	1.38 <sup>b</sup>	0.02	0.01	0.16	0.01
Valeric. %	1.05 <sup>b</sup>	1.26 <sup>a</sup>	0.01	0.05	0.95	0.15
<b>ADC</b>						
Dry matter	0.61	0.65	0.03	0.57	-	-
Organic matter	0.63	0.67	0.02	0.43	-	-
Crude protein	0.53 <sup>b</sup>	0.63 <sup>a</sup>	0.03	0.05	-	-
NDF	0.57	0.54	0.02	0.24	-	-
ADF	0.55	0.55	0.02	0.96	-	-
Ether extract	0.64	0.75	0.05	0.21	-	-

Note 1: Means followed by letters written differently (a, b) on the same line illustrate a treatment effect when considering  $p \leq 0.05$  and a tendency to be different when  $p > 0.05$  and  $\leq 0.10$ ; Note 2: Details of the treatment × day interaction and day effect were presented in Supplementary Material 6.

### 3.10. Apparent Nutrient Digestibility Coefficient

The diet digestibility results are presented in **Table 5** and **Table S4**. The treatment affected the CP's apparent digestibility coefficient, which was higher in cows that consumed BC ( $p \leq 0.05$ ). The treatment did not influence the digestibility of other nutrients (organic matter, fiber, EE— $p > 0.05$ ).

## 4. Discussion

Cows that consumed BC produced a greater quantity of milk compared to CON, although there was no effect of the treatment on the feed intake of the cows in the present study, an effect on consumption similar to that reported by Nunes *et al.* [1] in an experiment carried out with Holstein cows fed BC, which had 130 days of lactation and an average production of 27.6 liters. In a study with lactating ewes, greater milk production was also found in animals that consumed BC [3], similar

to that observed here. The greater milk production of cows fed BC is because the additive improved the digestibility of dietary fat [1], resulting in a greater quantity of fatty acids available to be absorbed and used by the body as energy. It is already known that in high-producing cows, the liver is one of the central organs that needs to be fully functioning when we seek to enhance productivity [27] [28]. In this sense, we understand that the source of choline used has a hepatoprotective effect; thus, more nutrients are metabolized and sent to the bloodstream. Choline acts as a methionine-sparing methyl donor in the metabolism of dairy synthesis [1], and according to Pinotti *et al.* [29], dietary supplementation of methyl donors in rumen-protected nutrients can improve methyl group preservation and methylneogenesis in lactating cows. It is also worth mentioning that choline can be considered an essential and limiting nutrient in milk synthesis because milk has many methylated compounds [30]. According to the literature, limited supply of methyl group additives such as methionine and choline may contribute to inadequate hepatic synthesis of phosphatidylcholine and hepatic export of triglycerides, systemic oxidative stress, and compromised milk production [31]. A recent study found that supplementing diets fed to transition cows with protected choline before and after calving increased plasma choline concentrations and reduced the risk of hepatic lipidosis [32], thus being beneficial to the health of cows as occurred in the present study.

In the present study, unlike other studies, a more significant amount of total solids was found in cows' milk that consumed BC. Deuchler *et al.* [33], providing 50 g of BC/cow/day, did not observe any effects on fat and protein concentrations in milk, just as Alba *et al.* [2] also did not observe any effect of BC on variables in the milk of dairy sheep. There is no definitive explanation for this difference between results for milk composition, but we suspect that different lactation periods, ration, and species may justify this difference between studies using BC. Our findings are desirable from the point of view of milk quality; therefore, we understand that dietary manipulation using BC can be a practice with rapid and efficient responses, which also alters the fatty acid profile in milk fat. It is important to remember that milk fat is the most accessible component for manipulation, as its production can be reduced by up to 50% or more when compared to protein and lactose contents [34]. However, in our study, there was also an increase in protein. Therefore, since the percentage of fat in milk was higher in cows that received a source of choline than in those that CONT, this is believed to be related to the greater ruminal production of acetic acid, which is essential for lipid metabolism [35]. According to the literature, the mammary gland increases the fat percentage through new synthesis at the time of synthesizing fatty acids that form milk fat [36].

Protein was also higher in the milk of cows in the treatment group compared to the control group. Few studies have demonstrated an increase in the percentage of protein in milk composition when additives are used [37]. Even so, we believe it is related to the better digestibility of CP. In the present study, we understand

that phosphatidylcholine had a hepatoprotective action, as it improved liver health and reduced the activity of extravasation enzymes, which indicate cell damage. We understand that the addition of BC improved liver functionality, favoring the metabolism of amino acid compounds released into the circulation, and consequently, this reflected in more significant protein in the milk.

The tendency of reduction of SCC in milk is a positive effect, considering milk quality. Since choline participates in cell formation, cell signaling, and tissue integrity, animals that consumed BC may present more resistant cell structures, with a consequent reduction in the desquamation of the secretory epithelium of the udder, thus reducing the SCC [38]. In addition, it is known that phosphatidylcholine has an anti-inflammatory effect [39], and this is why we expected that there would be a lower SCC, which was restricted to a numerical condition, with a trend. We believe that the results were promising, since we worked with a small sample size and a short experimental period. Therefore, if these conditions were greater, we could have statistically significant results, which need to be investigated in the future. However, we need to understand that feeding, bedding management and milking management are direct factors that can affect the health of the mammary gland, altering SCC; a reason that justifies caution in interpreting the data here.

The profile of fatty acids in milk in our study draws attention to an increase in unsaturated fatty acids, which can benefit the consumer, as they are desirable fats when we think about human health. According to the literature, the perspective of manipulating milk fat aims to meet the demand of a consumer market, which is increasingly resistant to the consumption of certain saturated fats due to their deleterious effects on human health, mainly related to the cardiovascular system [40]. The consumption of BC reduced two saturated fatty acids in milk (C15:0 and C17:0) and increased one saturated fatty acid C18:0. Furthermore, animals fed with BC had a greater production of omegas (oleic, linoleic, and  $\alpha$ -linolenic), which is desirable for human health, as linoleic acid, for example, is beneficial for maintaining under normal conditions, the cell membranes, brain functions and the transmission of nerve impulses, among others [41].

Further studies are needed to understand milk fatty acid profile modulation when BC is used. However, our central hypothesis is related to hepatic metabolism, which was favored by the availability of choline and thus had more effective participation in the metabolism of lipids in the animal organism and, consequently, in the product, in this case, milk. This is because, in our study, the variables ALT and AST showed lower activity in the treatment cows than in the control animal group. It is worth mentioning that the ALT enzyme was 12.5% lower in cows that consumed BC compared to the control group and AST, which was 13.85% lower. The increase in the activity of these liver enzymes is an essential diagnostic index in liver injuries and liver and biliary system overload, indicating hepatocyte rupture due to the progressive accumulation of lipid granules and, subsequently, increased permeability of these enzymes in the blood [42]. A study carried out in Iran with ewes in late gestation, with dietary restrictions combined

with the consumption of propylene glycol, sodium monensin, and choline chloride, observed a reduction in the value of AST and ALT in the blood on day 124 of gestation [43]. Alba *et al.* [2] performed a study with ewes in the transition period fed with 5 g/animal days and found no difference in the levels of AST and GGT during the first 30 days postpartum, but on day 45 postpartum, the activity of the enzymes was lower. BC has these liver-protective effects because it contains choline as choline conjugates, mainly BC. This emulsifying phospholipid has a detergent action and reduces surface tension, forming smaller fat particles as triglycerides. Therefore, this leads to a lower concentration of liver enzymes in the serum [44]. It is worth noting that the values indicated for Jersey cattle for AST enzymes are  $38.6 \pm 11.24$  [45], and ALT for cattle should be less than 40 U/L [46]. The effect of day on liver injury biomarkers in the control group was not expected, since the dose provided was within that established for lactating cows.

Glucose levels were lower in the serum of cows that consumed BC compared to the control group. However, the ideal parameters for dairy cattle present reference values between 45 mg/dL and 75 mg/dL [47]. The lower the glucose concentrations in the blood, the greater the glucose efficiency in the cell. According to Xu *et al.* [48], adding choline to the diet improves energy metabolism by making more glucose available to the cells. Bryant *et al.* [49] found that Suffolk lambs fed rumen-protected choline presented lower glucose levels than the control group, suggesting increased insulin production. Therefore, choline plays a fundamental role in lipid and glucose metabolism and synthesizes molecules responsible for the orientation and function of several intracellular signaling proteins [50].

The intake of the BC additive did not affect the white blood cell count but stimulated an increase in red blood cells. In a study with sheep in the transition period, Alba *et al.* [2] observed no difference between treatments in blood count variables. A possible hypothesis for the increase in erythrocyte count may be related to an indirect effect of lipid metabolism, as lipids can influence terminal erythropoiesis through the action of the enzyme phosphocholine phosphatase, which can regulate erythroblast proliferation, enucleation, and erythroid tissues [51], since this increase in red blood cells was observed at the end of the adaptation period (d14) and in the middle and end of the experimental period (d21 and d28). However, this needs to be investigated in the future, as well as the lower number of platelets seen in animals that consumed BC; however, within the reference values for the species (100 - 800 thousand cells/dL). In summary, the effect of BC on the blood count is positive, but we are not clear on the mechanisms involved.

In this study, it was found that BC intake reduces oxidative reactions, reducing lipid peroxidation because it stimulates the increase in the activities of antioxidant enzymes, such as SOD and GST, in the study. Similar results have already been described in other studies with cows [52], dairy sheep [2] [3], and young sheep [53]. The antioxidant effect of oral choline in the diet is widely discussed, but the mechanism involved in cows needs to be investigated in future studies. However, the increase in GST shows that the liver-protective enzyme is a strong ally in min-

imizing oxidative stress.

Unlike the study developed by Nunes *et al.* [1], which showed a higher digestibility coefficient of the EE, in our work, animals that consumed BC showed an improvement in the digestibility of CP. Possible reasons for this difference between the studies are the breed of the cow and also the number of lactations; remembering that we used primiparous Jersey cows; since these two situations strongly influence the cow's metabolism. Few studies have evaluated the effects of the digestibility of nutrients in the diet with choline of vegetable origin. Still, the process is believed to be related to ruminal fermentation and modulation of VFA. Due to the greater milk production, we expected a higher proportion of propionate, as well as glucose; however, the opposite occurred, that is, both were at lower values in the cows in the treatment group. In this study, there was a different modulation in the proportion of SCFA in the ruminal environment in the presence of BC and CONT.

## 5. Conclusion

Adding biocholine to the diet of lactating Jersey cows enhances milk production without altering feed intake. When cows consumed BC, their milk had a higher concentration of protein and fat, which was reflected in a higher percentage of total solids. BC intake modulated the fatty acid profile in milk, increasing the proportion of unsaturated fatty acids. BC intake improved the CP digestibility coefficient of the diet, which explains the effects on milk production and composition. BC intake reflected a reduction of extravasation enzymes that characterize hepatocyte injury and increased the GST activity, an important antioxidant enzyme for hepatic detoxification. Furthermore, we concluded that BC intake influences the oxidative status of cows, reducing lipid peroxidation and increasing the activity of antioxidant enzymes, which confirms the antioxidant effect for cows.

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## Ethics Committee

This work was submitted and approved by the Ethics Committee for the use of animals at UDESC under protocol number 8303290323, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA/Brazil).

## Data Availability

All data and materials used in the experiment are available and ready to be provided.

## Authors' Contributions

M Breancini and A.S. da Silva contributed to the research's design and implementation to analyze the results. R Wagner and GV Kozloski helped develop the project and its execution and financing. MG Vitt, MH Signor, GJ Wolschick, RVP Lago, NG Correa, KW Leal, and ALR Brunetto participated in the execution of the experiment and collection of samples and data. LEL Silva and CTK Jung performed the laboratory analysis. All authors discussed the results and contributed to the final manuscript.

## Consent to Participate

All names in the author list were involved in various stages of experimentation or writing.

## Consent for Publication

All authors agree to submit the paper for publication in the journal.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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## Supplementary Material

**Table S1.** Standardization of measurement of short-chain fatty acids in rumen fluid.

Item	Acetic acid	Propionic acid	Butyric acid	Isovaleric acid
R <sup>2</sup>	0.9998	0.9994	0.9998	0.9998
Equation	$y = 0.0117x + 0.0129$	$y = 0.0203x + 0.0059$	$y = 0.0285x - 0.0019$	$y = 0.0371x - 0.0022$
Linear range (mmol·L <sup>-1</sup> )*	2.30 - 138.26	1.76 - 105.49	1.35 - 80.86	0.57 - 34.29
LOD (mmol·L <sup>-1</sup> )	1.15	0.88	0.67	0.29
LOQ (mmol·L <sup>-1</sup> )	2.30	1.76	1.35	0.57
Accuracy (%)	107.8	109.3	107.7	104.8
Repeatability (RSD)	5.67	2.70	2.48	2.55

\*The linear range, LOD and LOQ were expressed in mmol of SFA for L of ruminal fluid.

**Table S2.** Effect of day and treatment vs. day interaction on milk composition and fatty acid from cows fed biocholine (treatment group) compared to control group.

Variables	Control	Treatment	SEM	P: day	P: treat × day
Fat (g/100g)				<b>0.03</b>	<b>0.05</b>
d1	4.29	4.34 <sup>B</sup>	0.13		
d14	4.08 <sup>b</sup>	4.77 <sup>aAB</sup>	0.16		
d21	4.25	4.58 <sup>AB</sup>	0.14		
d25	4.78	4.79 <sup>AB</sup>	0.15		
d28	4.72 <sup>b</sup>	5.20 <sup>aA</sup>	0.13		
Protein (g/100g)				<b>0.01</b>	<b>0.04</b>
d1	3.86 <sup>A</sup>	3.71	0.06		
d14	3.22 <sup>bb</sup>	3.78 <sup>a</sup>	0.11		
d21	3.61 <sup>A</sup>	3.76	0.19		
d25	3.46 <sup>AB</sup>	3.71	0.07		
d28	3.60 <sup>A</sup>	3.88	0.12		
Lactose (g/100g)				0.12	<b>0.01</b>
d1	5.13	5.11	0.11		
d14	4.63 <sup>b</sup>	5.36 <sup>a</sup>	0.14		
d21	5.14	5.34	0.15		
d25	4.93 <sup>b</sup>	5.20 <sup>a</sup>	0.10		
d28	4.84	4.92	0.10		

**Continued**

Total solids (g/100g)				<b>0.04</b>	<b>0.02</b>
d1	13.3 <sup>A</sup>	13.2 <sup>B</sup>	0.10		
d14	11.9 <sup>bb</sup>	13.9 <sup>aa</sup>	0.14		
d21	13.0 <sup>A</sup>	13.7 <sup>A</sup>	0.14		
d25	13.2 <sup>A</sup>	13.7 <sup>A</sup>	0.11		
d28	13.2 <sup>A</sup>	14.0 <sup>A</sup>	0.11		
C15:0 (Pentadecanoic)				0.05	0.01
d0	0.92 <sup>B</sup>	0.99	0.02		
d14	1.67 <sup>aa</sup>	1.20 <sup>b</sup>	0.04		
d28	1.37 <sup>ab</sup>	1.22 <sup>b</sup>	0.03		
C18:0 (Stearic)				0.22	0.10
d0	12.6	11.9	0.36		
d14	10.1	9.90	0.32		
d28	9.50 <sup>b</sup>	10.7 <sup>a</sup>	0.31		
C18:2n6c (Linoleic)				0.01	0.01
d0	2.07	2.12 <sup>B</sup>	0.06		
d14	2.04 <sup>b</sup>	3.02 <sup>aa</sup>	0.05		
d28	1.89 <sup>b</sup>	3.95 <sup>aa</sup>	0.05		
Σ Unsaturated fatty acids (UFA)				0.02	0.01
d0	23.4 <sup>A</sup>	23.1 <sup>B</sup>	0.14		
d14	21.6 <sup>bb</sup>	23.6 <sup>aaB</sup>	0.11		
d28	21.2 <sup>bb</sup>	24.0 <sup>aa</sup>	0.12		

Note 1: Day effect was verified when  $p < 0.05$ , illustrated by different capital letters in the same column (A, B); Note 2: Treatment  $\times$  day interaction was verified when  $p < 0.05$ , illustrated by lowercase letters in the same row (a, b).

**Table S3.** Effect of day and treatment vs. day interaction on blood count and serum biochemistry of cows fed with biocholine (treatment group) compared to control group.

Variables	Control	Treatment	SEM	P: day	P: treat $\times$ day
Erythrocytes ( $\times 10^6/\mu\text{L}$ )				0.17	0.01
d1	5.43	5.85	0.12		
d14	5.37 <sup>b</sup>	6.06 <sup>a</sup>	0.10		
d21	5.37 <sup>b</sup>	5.85 <sup>a</sup>	0.10		
d28	5.55 <sup>b</sup>	6.09 <sup>a</sup>	0.11		

**Continued**

Hemoglobin (mg/dL)				0.01	0.01
d1	9.69	10.3 <sup>B</sup>	0.14		
d14	9.09 <sup>b</sup>	9.73 <sup>aB</sup>	0.13		
d21	9.37 <sup>b</sup>	9.93 <sup>Ab</sup>	0.12		
d28	9.86 <sup>b</sup>	13.0 <sup>aa</sup>	0.18		
Hematocrit (%)				0.83	0.02
d1	26.8	27.0	0.32		
d14	25.5 <sup>b</sup>	27.2 <sup>a</sup>	0.25		
d21	26.2	27.3	0.31		
d28	26.5 <sup>b</sup>	28.1 <sup>a</sup>	0.23		
Platelets ( $\times 10^3/\mu\text{L}$ )				0.01	0.01
d1	296 <sup>B</sup>	309 <sup>A</sup>	30.4		
d14	288 <sup>B</sup>	252 <sup>B</sup>	21.7		
d21	337 <sup>aA</sup>	264 <sup>bB</sup>	16.6		
d28	314 <sup>aAB</sup>	192 <sup>bC</sup>	27.3		
ALT (U/L)				<b>0.01</b>	<b>0.01</b>
d1	27.5 <sup>B</sup>	27.7	0.63		
d14	33.0 <sup>A</sup>	30.4	0.72		
d21	31.4 <sup>aA</sup>	26.8 <sup>b</sup>	0.92		
d28	34.1 <sup>aA</sup>	27.0 <sup>b</sup>	0.74		
AST (U/L)				<b>0.01</b>	<b>0.01</b>
d1	94.4 <sup>B</sup>	100	2.58		
d14	124 <sup>A</sup>	118	4.77		
d21	127 <sup>aA</sup>	107 <sup>b</sup>	5.11		
d28	140 <sup>aA</sup>	110 <sup>b</sup>	4.64		
Uric acid (mg/dL)				0.04	<b>0.05</b>
d1	0.90	0.80 <sup>B</sup>	0.04		
d14	0.93 <sup>b</sup>	1.04 <sup>aA</sup>	0.06		
d21	0.80 <sup>b</sup>	1.07 <sup>aA</sup>	0.07		
d28	0.89	0.77 <sup>B</sup>	0.03		

**Continued**

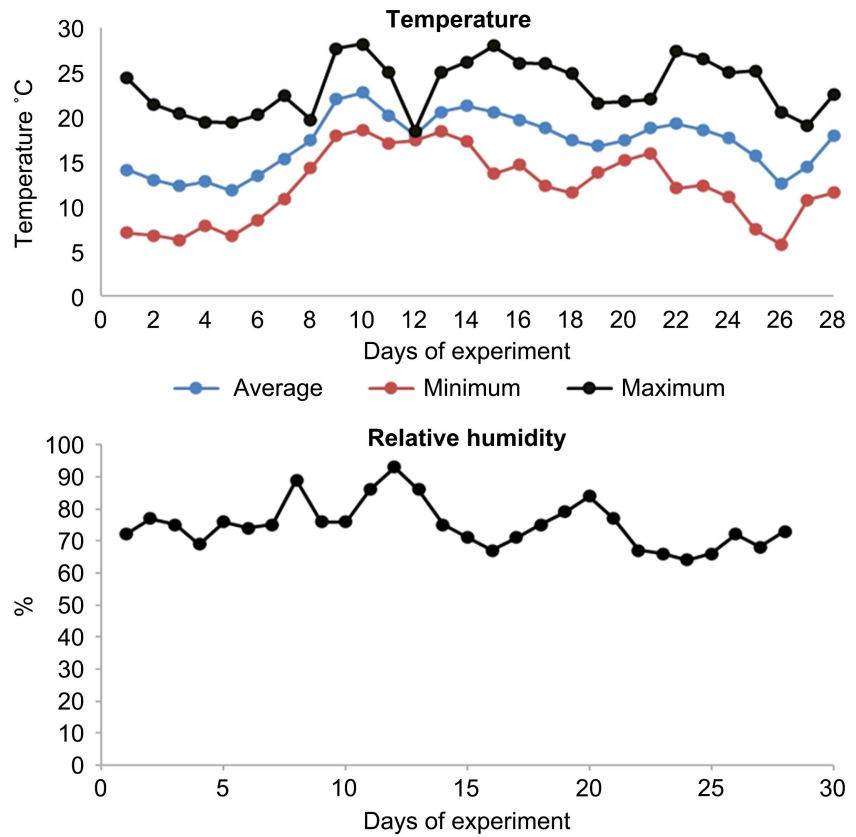
Glucose (mg/dL)				0.01	0.05
d1	54.0 <sup>C</sup>	57.8 <sup>B</sup>	1.40		
d14	55.1 <sup>C</sup>	52.8 <sup>B</sup>	1.21		
d21	63.0 <sup>aB</sup>	56.4 <sup>bB</sup>	1.75		
d28	73.8 <sup>aA</sup>	66.7 <sup>bA</sup>	1.46		
GST (U GST/mg protein)				0.01	0.01
d1	235	228 <sup>C</sup>	2.95		
d14	230	241 <sup>B</sup>	3.45		
d21	238 <sup>b</sup>	259 <sup>Aa</sup>	4.68		
d28	233 <sup>b</sup>	256 <sup>aA</sup>	4.66		
SOD (U SOD/mg protein)				0.01	0.01
d1	5.63	5.42 <sup>B</sup>	0.24		
d14	5.32	5.38 <sup>B</sup>	0.35		
d21	6.41	6.54 <sup>B</sup>	0.36		
d28	5.57 <sup>b</sup>	7.69 <sup>aA</sup>	0.40		

Note 1: Day effect was verified when  $p < 0.05$ , illustrated by different capital letters in the same column (A, B); Note 2: Treatment  $\times$  day interaction was verified when  $p < 0.05$ , illustrated by lowercase letters in the same row (a, b).

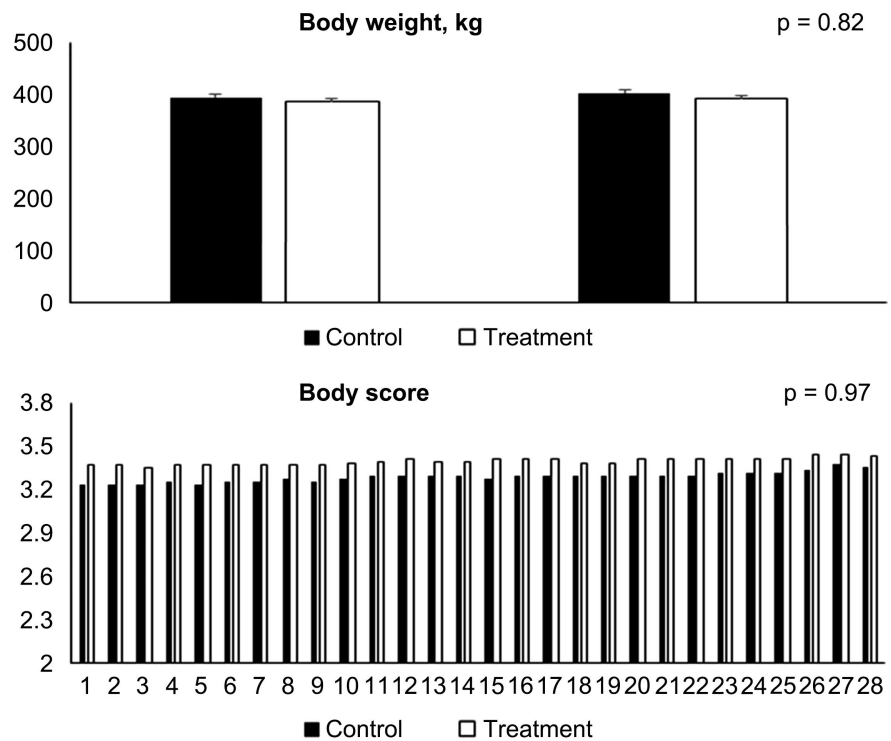
**Table S4.** Effect of day and treatment vs. day interaction on profile of SCFAs in the rumen fluid of cows that consumed biocholine.

Fatty acid	Control	Treatment	SEM	P: day	P: treat $\times$ day
Acetic %				0.95	0.02
d14	63.9 <sup>b</sup>	66.8 <sup>a</sup>	0.61		
d28	64.2 <sup>b</sup>	66.3 <sup>a</sup>	0.59		
Propionic %				0.97	0.01
d14	22.8 <sup>a</sup>	18.3 <sup>b</sup>	0.21		
d28	22.2 <sup>a</sup>	18.5 <sup>b</sup>	0.23		
Butyric. %				0.93	0.01
d14	10.4 <sup>b</sup>	13.1 <sup>a</sup>	0.17		
d28	10.5 <sup>b</sup>	12.3 <sup>a</sup>	0.17		
Isovaleric. %				0.16	0.01
d14	1.79 <sup>a</sup>	1.29 <sup>b</sup>	0.02		
d28	1.87 <sup>a</sup>	1.48 <sup>b</sup>	0.01		

Note 1: Means followed by letters written differently (a, b) on the same line illustrate the difference between groups when considered  $p \leq 0.05$ .



**Figure S1.** Temperature and humidity in the experimental unit during the research.



**Figure S2.** Body weight (digital scale) and body score (camera attached to the robot) of Jersey cows fed with biocholine (treatment group) and monensin (control group).