

LUISA FERNANDA LONDOÑO SALAZAR

**EFFECT OF SELECTED FEED ADDITIVES TO IMPROVE GROWTH AND
HEALTH OF DAIRY CALVES**

Dissertation presented to the Federal University of Viçosa, as part of the requirements of the Graduate Program in Animal Science, to obtain the title of Magister Scientiae.

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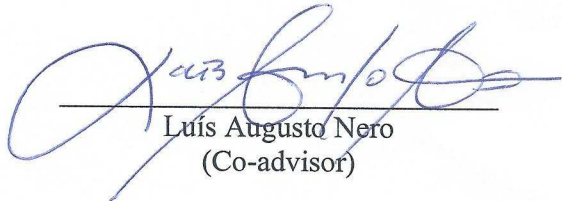
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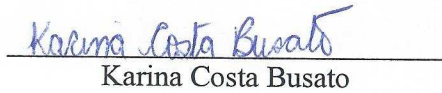
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“NO TE RINDAS, POR FAVOR NO CEDAS, AUNQUE EL FRIO
QUEME, AUNQUE EL MIEDO MUERDA, AUNQUE EL SOL SE
ESCONDA, Y SE CALLE EL VIENTO, AUN HAY VIDA EN TUS
SUEÑOS. PORQUE LA VIDA ES TUYA Y TUYO TAMBIEN EL DESEO,
PORQUE CADA DIA ES UN COMIENZO NUEVO, PORQUE ÉSTA ES
LA HORA Y EL MEJOR MOMENTO”

Mario Benedetti

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BIOGRAPHY

Luisa Fernanda Londoño Salazar, daughter of Gabriel Mario Londoño Pulgarin and Claudia Elena Salazar Medina, was born on February 21, 1990, in Medellín, Colombia.

In August 2008, she joined the University of Antioquia, finishing the undergraduate course in Animal Science in December 2014.

She began her master's degree in Animal Science by the Federal University of Viçosa in March 2015, submitting herself to the dissertation defense on July 21, 2017.

SUMMARY

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ABSTRACT

SALAZAR, Luisa Fernanda Londoño, M.Sc., Universidade Federal de Viçosa, July, 2017
Effects of selected feed additives to improve growth and health of dairy calves.
Adviser: Marcos Inácio Marcondes. Co-advisors: Polyana Pizzi Rotta Costa e Silva and
Luís Augusto Nero.

We aimed to evaluate the effect of supplementation with monensin (MON), probiotics or essential oils on performance and fecal consistency index (FCI) of suckling dairy calves from 6-60 d of age, and its residual effect 15 d after weaning. Fifty Holstein calves were fed 5 L of raw milk per day plus starter concentrate until weaning. The treatments were: Control (CON), addition of MON (30 mg/kg of starter), probiotic *E. faecium* (PROB, 70 mg/kg of starter, CFU/kg 7,0E+09, Cylactin[®]), essential oils (EO, 300 mg/kg of starter, CRINA[®] Ruminants), or addition of PROB +EO (EOPROB). DMI and fecal score (scored from 1 to 4) were daily measured, and animals were weighed every 15 d. DNA extraction from feces was performed, to identifying by PCR the presence or absence of microorganisms (*E. coli*, *Hafnia*, *Shiguella*, *Lactobacillus spp*, *Enterococcus spp*, and *Enterococcus faecium* NCIMB 10415) in the feces. Two 72 h digestibility trials were performed at days 20-28 (period 1) and 50-56 (period 2), by total fecal collection. The experiment was designed in completely randomized block with ten replications per treatment, and date of entrance used as block. ANOVA was performed to test treatment effects at $P < 0.10$, means were compared by Student's t-test, and PCR results were analyzed by Wald test. The dry matter intake (DMI) before weaning was greater ($P < 0.05$) for EO (903.03 g / d) compared to MON (794.34 g / d) and EOPROB (783.12 g / d). EO and MON decreased ($P < 0.012$) FCI during pré-weaning. Average daily gain (ADG) and feed efficiency (FE) did not differ among treatments ($P > 0.10$) before weaning. The withers height (WH) and croup height (CH) was no different among treatments after or before weaning. After weaning the DMI and FCI did not differ among

treatments ($P > 0.10$). EO had greater ($P < 0.05$) ADG (917.50 g / d) compared to CON (615.80 g/d) and PROB (592.60 g / d). The FE improved ($P < 0.05$) with EO (0.72 g / g) over CON (0.36 g / g), MON (0.49 g / g) and PROB (0.36 g / g). The PCR results showed absence of *E. faecium* NCIMB 10415 in animals fed PROB and CON. During the digestibility trials, greater intakes ($P < 0.001$) of nutrients were observed at days 50-56 compared to 20-28 days. Animals fed PROB had greater ($P < 0.05$) intakes of DM (1184.56 g / d), crude protein (CP) (254.63 g /d) and neutral detergent fiber (NDF) (320.36 g / d) than animals fed EOPROB. Intake of Non-fibrous carbohydrates (NFC) were greater ($P < 0.05$) in EO (384.31 g / d) compared to CON (323.63 g / d), MON (323.84 g / d) and EOPROB (301.30 g / d). The ether extract (EE) and organic matter (OM) intake was not affected ($P > 0.10$) by treatments. NDF digestibility was greater ($P < 0.012$) in animals fed MON (924 g / d). In conclusion, EO can be added to the dairy calf ration to improve fecal score, increases DMI and improves nutrient digestibility. The pre-weaning FCI decreased with MON and increased with PROB.

RESUMO

SALAZAR, Luisa Fernanda Londoño, M.Sc., Universidade Federal de Viçosa, julho de 2017. **Suplementação de Monensina, óleos essenciais e probiótico *Enterococcus faecium* NCIMB 10415 para bezerros lactentes.** Orientador: Marcos Inácio Marcondes. Coorientadores: Polyana Pizzi Rotta Costa e Silva e Luís Augusto Nero.

Objetivou-se avaliar o efeito da suplementação de monensina (MON), probióticos ou óleos essenciais sobre o desempenho e índice de escore fecal (IEF) dos bezerros leiteiros de 6-60 d de idade e seu efeito residual 15 dias após o desmame. Cinquenta bezerros da raça Holandesa foram alimentados com 5 L de leite cru por dia mais concentrado inicial até o desmame. Os tratamentos foram: Controle (CON), adição de MON (30 mg / kg de concentrado), probiótico *E. faecium* (PROB, 70 mg / kg de concentrado, CFU / kg 7,0E + 09, Cylactin®), óleos essenciais (OE, 300 mg / kg de concentrado, CRINA® Ruminants), ou adição de PROB + OE (EOPROB). O consumo do alimento e o escore fecal (pontuado de 1 a 4) foram medidos diariamente e os animais foram pesados a cada 15 dias. Foi realizada a extração de DNA das fezes, para identificar por meio da PCR a presença ou ausência de microorganismos (*E. coli*, *Hafnia*, *Shiguella*, *Lactobacillus spp*, *Enterococcus spp* e *Enterococcus faecium* NCIMB 10415). Dois ensaios de digestibilidade de 72 h foram realizados nos dias 20-28 (período 1) e 50-56 (período 2), por coleta fecal total. O experimento foi definido em blocos inteiramente casualizados com dez repetições por tratamento e data de entrada usada como bloco. A ANOVA foi realizada para testar os efeitos do tratamento em $P < 0,10$, as medias foram comparadas pelo teste t de Student e os resultados da PCR foram analisados pelo teste de Wald. O consumo de matéria seca (CMS) antes do desmame foi maior ($P < 0,05$) para OE (903,03 g / d) em comparação com MON (794,34 g / d) e EOPROB (783,12 g / d). Animais OE e MON diminuíram ($P < 0,012$) o IEF durante o aleitamento. O ganho médio diário (GMD) e a eficiência alimentar (EA) não diferiu ($P > 0,10$) entre os tratamentos antes do desmame. A altura à cernelha (AC) e altura à garupa (AG) não foi diferente entre os

tratamentos ($P > 0,10$) antes nem depois do desaleitamento. Após o desmame, o CMS e IEF não diferiu entre os tratamentos ($P > 0,10$). OE apresentou maior GMD (917,50 g / d) ($P < 0,05$) em comparação com CON (615,80 g / d) e PROB (592,60 g / d). A EA melhorou ($P < 0,05$) com OE (0,72 g / g) sobre o CON (0,36 g / g), MON (0,49 g / g) e PROB (0,36 g / g). Os resultados de PCR mostraram ausência do *E. faecium* NCIMB 10415 em animais PROB e COM. Durante os ensaios de digestibilidade, houve maior consumo ($P < 0,001$) de nutrientes nos dias 50-56 em comparação com os dias 20-28. Animais PROB apresentaram maiores ($P < 0,05$) consumo de MS (1184,56 g / d), proteína bruta (PC) (254,63 g / d) e fibra detergente neutra (FDN) (320,36 g / d) comparado com EOPROB. A ingestão de carboidratos no fibrosos (CNF) foi maior ($P < 0,05$) no tratamento OE (384,31 g / d) comparado com CON (323,63 g / d), MON (323,84 g / d) e EOPROB (301,30 g / d). A ingestão de extrato etéreo (EE) e matéria orgânica (MO) não foi afetada ($P > 0,10$) pelos tratamentos. A digestibilidade da FDN foi maior ($P < 0,012$) em animais alimentados com MON (924 g / d). Em conclusão, OE pode ser adicionado à ração de bezerros leiteiros para melhorar o escore fecal, aumenta o CMS e melhora a digestibilidade dos nutrientes. O IEF no pré-desmame diminuiu com MON e aumentou com PROB.

1. INTRODUCTION

During the first days of life, rumen and microbial population are not completely developed and functional, and stress factors like dehorn, weaning, vaccination, or extreme changes of temperature, might cause decreased immunity, consequent diarrheas, then weight loss or reduced performance (Cho and Yoon, 2014). Producers often use antibiotics as prevention and treatment of diarrhea in calves, which is why there has been great concern worldwide with food security, since it is possible to observe residual medicaments in food of animal origin, affecting human health in the form of allergies, intoxication and inducing antimicrobial resistance in bacteria both in the animal and in humans (Fey et al., 2000; FAO, 2004; Khachatryan et al., 2004).

The monensin is an ionophore antibiotic, which has the ability to form liposoluble complexes with sodium and potassium ions in the microorganisms, increasing the permeability of the cellular membranes to such ions, promoting an osmotic imbalance, increased energy expenditure and subsequent cell death (Russell and Strobel, 1989). Thus, monensin exhibits anticoccidial and antibacterial properties, used commercially as a coccidiostat for poultry and as a growth promoter for ruminants (Stephan et al., 1997; Butaye et al., 2003; Huczyski et al., 2013). However, the use of ionophores in livestock production is banned in the European Union (Union, 2003) due to development of monensin resistance by some bacteria (Newbold et al., 1993; Russell and Houlihan, 2003).

As alternative to antibiotics, the probiotics have been used, which have beneficial effects on gastrointestinal tract, for instance via modulation of the immune system (Restani and Barrett, 2003) and decrease the incidence of diarrheas (Taras et al., 2006), however the results are variable. Probiotic strains of *Enterococcus faecium* have been very studied in piglets, with positive impact on intestinal microbiota, reflected in the

reduction of enteropathogenic bacterial load of suckling piglets fed *E. faecium* SF68 (Scharek et al., 2005), and in the presence of the probiotic in the feces during the whole period of supplementation (Starke et al., 2013). In contrast, (Kreuzer et al., 2012)), did not find beneficial effects in weaned pigs fed *Enterococcus faecium* NCIMB 10415. To our knowledge there are no data in the literature on the effects of *Enterococcus faecium* NCIMB 10415 on dairy calves.

More recently, essential oils (EO) have also been widely used as a new class of feed additive to improve the intestinal microbiota of domestic animals (Michiels et al., 2009; Brenes and Roura, 2010). The EO are a mixture of many chemical compounds (mainly terpenes and terpene derivatives) (Baser and Buchbauer, 2010) responsible for an antimicrobial activity, due to their chemical structure, causing alteration on membrane structure and increasing permeability (Šarac et al., 2014). Generally, EO are more effective against Gram-positive than Gram-negative bacteria (Nagy and Tengerdy, 1968; Trombetta et al., 2005) since the structure of gram-positive bacteria cell wall allows hydrophobic molecules to easily penetrate the cell, and to act both on cell wall and within the cytoplasm, while the gram-negative bacteria are more resistant, due to the lipopolysaccharides contained in their outer membrane (Nazzaro et al., 2013). However, carvacrol, eugenol and thymol, are capable of disintegrating the outer membrane of Gram-negative bacteria such as *Escherichia coli* and *Salmonella* Typhimurium (Stein and Kil, 2006). In addition, it is also suggested that the addition of EO in the concentrate can increase dry matter intake (DMI) and average daily gain (ADG) of Holstein calves (Jeshari et al., 2016).

Finally, alternatives such as probiotics and EO must be studied as replacement for monensin. Therefore, we hypothesized that the addition of probiotics, EO or monensin, may improve the performance and intestinal environment in calves, reducing the

incidence of diarrhea. We also hypothesized that the combined use of EO and probiotics will increase the beneficial aspects of these additives when used separately.

The objective of this study was to evaluate the effect of Monensin, essential oils or probiotic *Enterococcus faecium* NCIMB 10415, or on DMI, ADG, digestibility, feces score and faecal microbial population of suckling calves from 6 to 60 days of age, and their residual effect 15 days after weaning.

2. MATERIALS AND METHODS

The experiment was carried out at the Teaching, Research and Extension Unit in Dairy Cattle of the Department of Animal Science of the Federal University of Viçosa-UFV (Viçosa, MG, Brazil), and it was approved by the Ethics Committee in the Use of Production Animals of the UFRV, process No. 26/2017.

Animals, feeding and treatments

Fifty six-day-old Holstein calves, with an average initial body weight of 38 ± 1.0 kg (mean \pm SE), were fed 5 L/d of raw milk, divided into 2.5 L in the morning (7:00 h) and 2.5 L in the afternoon (15/30 h), and a starter feed formulated with 60% whole ground corn, 20% soybean meal and 20% mineral core, containing the treatments: control (CON) without supplementation, probiotic *Enterococcus faecium* NCIMB 10415 (PROB, 70 mg/kg of starter, CFU/kg $1.4E+09$, Cylactin[®]), essential oils (EO, 300 mg/kg of starter; blend of thymol, guaiacol, eugenol, vanilin, salicylaldehyde and limonene, (Vendramini et al., 2007) Crina Ruminants[®]), probiotic + essential oils (EOPROB, treatments PROB+EO), and monensin (MON, 30 mg/kg of starter). The supplements were provided by DSM Nutritional products Ltd., Brasil. The animals were supplemented from the 6th day of life to weaning (60 days of life), then all animals were fed 2000 g/d of the control treatment and corn silage *ad libitum* separately, for 15 more days. The starter and clean

water were offered in separate buckets always in the morning.

Measures of intake, weight, fecal score and digestibility trial

The intake of starter and silage were measured every morning, as the difference between the amount offered and the leftovers. The animals were weighed and measured at withers height (WH) and at croup height (CH), at beginning of the experiment, then every 15 days, at weaning and at the end of the experiment.

The animals' faecal consistency score was daily monitored (scored from 1 to 4 where 1 = pasty; 2 = pulpy; 3 = soupy; 4 = watery) (Wenge et al., 2014). Calves with fecal consistency 3 or 4 were classified as having diarrhea. When the animal presented pale and dry mucous membranes along with diarrhea, the intervention with hydration was required: 8 g of common salt (NaCl), 8 g of NaHCO₃, 2g of KCl, 15g of dextrose and 2 L of warm water. The Fecal Consistency Index (FCI) was determined according to the following equation (Passini et al., 2001):

$$FCI = \frac{[(dS1 \times 1) + (dS2 \times 2) + (dS3 \times 3) + (dS4 \times 4)]}{Td \times 4} \times 100$$

Where, dS1, dS2, dS3, dS4 represent the number of days with fecal consistency of score 1, 2, 3, 4, respectively; and Td represents the total days.

For the digestibility trials, healthy animals were selected, at least three for each treatment. The animals were submitted to two 72h digestibility trials at days 20-28 (period 1) and 50-56 (period 2), with total fecal collection.

Chemical analysis

Samples of feces were partially dehydrated in a forced-ventilation oven (55°C) during 72 h and subsequently ground to 1 mm. Samples of milk were partially dehydrated by lyophilization and ground to 1 mm in a knife mill (Detmann et al., 2012). Samples of feeds and feces were evaluated for dry matter (DM) quantified by AOAC (2005) method

930.15 and organic matter (OM) by method 930.15. Nitrogen content was quantified by AOAC (2005) method 984.13, considering 6.25 as the constant factor to convert nitrogen values to crude protein (CP). The ether extract (EE) was evaluated according to AOCS (2009), and neutral detergent fibre (NDF) content was quantified with a heat stable amylase and sodium sulphite (Mertens, 2002), and Non-fibrous carbohydrate (NFC) calculated as $[100 - (NDF + CP + EE + \text{ash})]$.

DNA extraction and PCR from fecal samples

In the morning of days 5 (before the beginning of the supplementation), 30, 60 (weaning day) , and 70 (last day of experiment) fecal samples (50 g approximately) were collected by directly from the rectum. Each sample was homogenized with a sterilized spatula, then was weighed between 180 and 220 mg of the homogenized stool sample in eppendorf, and stored in an ultra-freezer at -80°C .

Extraction of DNA was performed in frozen feces samples, contained in the eppendorfs, using the QIAamp DNA Stool Mini Kit, following the manufacturer's recommendations. After extracting the DNA, 2 μL of all samples were analyzed in NanoDrop Lite (Thermo Scientific) to perform DNA quantification. After these procedures, the samples were stored at -20°C for subsequent analyzes.

The conventional PCR was performed in the Molecular Biology Laboratory (BIOMOL) of UFV, in order to identify the presence in the faeces of *E. coli*/*Hafnia*/*Shiguella* (Entero), *Lactobacillus spp* (Lac), *Enterococcus spp* (Ent) and *E. faecium* NCIMB 10415 (Cyl) after the treatment with the additives, using species-specific PCR primers according to Starke et al., (2013). Amplification reactions were conducted in a total volume of 25 μL containing: 12.5 μL of GoTaq® Green Master Mix 2X (Promega Corp), with 0.5 μL of each primer (10 mM / μL) (Table 1) and 9.5 μL of nuclease free water. Reactions were performed under the following conditions: For Enterobacter and

Lactobacillus; initial denaturation at 95° C for 2 minutes, followed by 35 cycles of denaturation at 94° C for 30 seconds, annealing at 58°C for 30 seconds, extension of 72°C per 1 minute and final extension of 72° C for 5 minutes; and for *Enterococcus spp* and *E. faecium* NCIMB 10415 initial desnaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension of 72°C per 1 minute and final extension of 72°C for 5 minutes. The PCR products were electrophoresed on a 1.5% agarose gel in Tris/borate/EDTA (TBE) buffer. The products were visualized by staining with UniSafe Dye-Uniscience (0.5 µg/mL).

Table 1. Primer Sequence, product length (base pairs-bp), and annealing temperature (°C) used for PCR in calves receiving mineral supplementation with monensin (MON), probiotic *Enterococcus faecium* NCIMB 10415 (PROB), Essential oils (EO), Probiotic + Essential oils (EOPROB), or mineral control (CON).

Specificity (Target name)	Primer name	Sequence	Product length[bp]	Annealing temperature [°C]
<i>E. coli/ Hafnia/ Shiguella</i> (16S rRNA)	Entero-F	GTAAATACCTTTGCTCATTGA	340	58
	Entero-R	ACCAGGGTATCTAATCCTGTT		
<i>Lactobacillus spp.</i> (16S rRNA)	LAC-1	AGCAGTAGGGAATCTTCCA	341	58
	LAC-2	CACCGCTACACATGGAG		
<i>Enterococcus spp.</i> (16S rRNA)	Ent1	CCCTTATTGTTAGTTGCCATCATT	144	61
	Ent2	ACTCGTTGTACTTCCCATTGT		
<i>E. Faecium</i> NCIMB10415 (plasmid)	Cyl-1a	TCGGAATTTGCCAGAGAGAC	208	60

(Starke et al., 2013)

Statistical analysis

The data of performance, DMI, FCI and feed efficiency (FE) were analyzed according to a completely randomized block, with the animals being blocked by gender and entrance to the experiment (2 blocks). Digestibility data of DM, protein and NDF

were evaluated as described above, but including the period as a time-repeated measure. Moreover, an additional FCI analysis was carried out, including the week of life as repeated measures to verify critical diverging moments between treatments. Period (digestibility trials) or week (fecal consistency) were included as fixed effect and the different residual variances were modeled using command REPEAT. All procedures were performed using PROC MIXED (SAS University Edition), being adopted the first order heterogeneous autoregressive matrix [ARH (1)] as matrix of variance and covariance. For the analysis of PCR data, a Wald Test was performed to determine difference between treatments. The comparisons between treatments were performed with the comparison of least square means by students 't' test at $P < 0.10$.

3. RESULTS

Performance parameters:

Before weaning, DMI was greater ($P < 0.05$) for EO (903.03 g/d) compared to MON (749.34 g/d) and EOPROB (783.12 g/d) (table 2), while no difference were found for PROB and CON groups (843.94 g/d and 845.05 g/d, respectively). The ADG, feed efficiency (FE), WH and CH did not differ ($P > 0.10$) among treatments. After weaning, DMI, WH and CH was not affected ($P > 0.10$) by the treatments. Calves from EO (917.50 g/d) group presented a greater ($P < 0.05$) ADG than those from PROB and CON groups (592.60 g/d and 615.80 g/d, respectively), while MON and EOPROB groups had similar ADG after weaning. The FE of EO (0.72 g/g) improved ($P < 0.05$) over control (0.36 g/g), MON (0.49 g/g) and PROB (0.36 g/g).

Table 2. Average daily gain (ADG, g/d), dry matter intake (DMI, g/d), feed efficiency (FE, g/g) and faecal consistency index (FCI) of Holstein calves fed different additives in the concentrate: EO (Essential Oils), PROB (probiotic *Enterococcus faecium* NCIMB 10415), MON (monensina), EOPROB (EO + PROB), CON (Control).

Item	Treatment					Standard Error	P value
	CON	MON	PROB	EO	EOPROB		
<i>Before weaning</i>							
DMI g/d	845.05 ^{ab}	749.34 ^b	843.94 ^{ab}	903.03 ^a	783.12 ^b	43.64	0.046
ADG g/d	663.60	605.10	680.80	616.00	602.20	70.21	0.384
FE g/g	0.77	0.76	0.82	0.68	0.75	0.08	0.319
FCI	0.66 ^a	0.59 ^b	0.71 ^a	0.59 ^b	0.65 ^a	0.02	0.012
WH cm/d	0.2833	0.2699	0.3202	0.2661	0.2456	0.034	0.608
CH cm/d	0.3362	0.3422	0.3860	0.3060	0.3168	0.032	0.455
<i>After weaning</i>							
DMI g/d	1637.48	1538.26	1590.80	1470.73	1380.68	134.46	0.171
ADG g/d	615.80 ^b	733.20 ^{ab}	592.60 ^b	917.50 ^a	794.70 ^{ab}	103.51	0.023
FE g/g	0.36 ^c	0.49 ^{bc}	0.36 ^c	0.72 ^a	0.60 ^{ab}	0.07	0.001
FCI	0.78	0.79	0.77	0.76	0.79	0.04	0.651
WH cm/d	0.2583	0.1686	0.2815	0.2444	0.3267	0.05	0.277
CH cm/d	0.2333	0.1629	0.2432	0.3259	0.2688	0.05	0.281

a,b,c :Means in a same row with different superscripts are significantly different ($p < 0.05$).

Fecal score:

Before the weaning, calves from EOPROB, PROB and CON groups presented similar FCI (0.65, 0.71 and 0.66, respectively, table 2); and calves fed PROB treatment presented greater ($P < 0.012$) incidence of diarrheas. In contrast, animals fed EO and MON had similar FCI (0.59), which was lower ($P < 0.05$) than other treatments, with low incidence of diarrheas. After weaning, FCI did not differ among treatments ($P > 0.10$). The weekly data analysis of fecal consistency (Figure 1), revealed that the effects observed previously were mainly due to an increase in the fecal score of the PROB fed animals and reduction of the score in animals fed EO and MON from weeks 4 to 6 ($P < 0.05$). There was a continuous increase in the fecal score of the animals over the weeks

of life, and at week 10 there was a reduction of animal fecal scores.

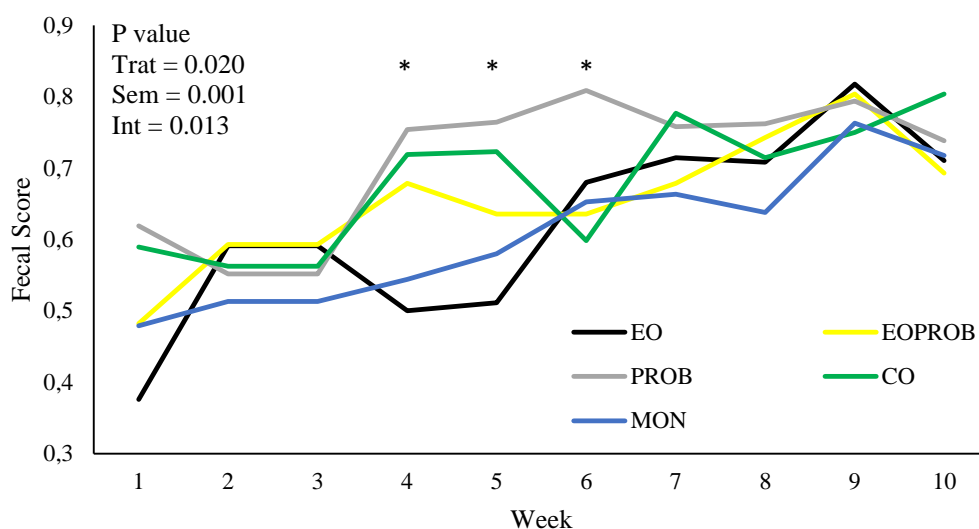


Figure 1. Fecal score of Holstein calves fed different additives in the concentrate: EO (Essential Oils), PROB (probiotic *Enterococcus faecium* NCIMB 10415), MON (monensina), EOPROB (EO + PROB), CON (Control). *Indicates significance ($P < 0.10$).

Digestibility:

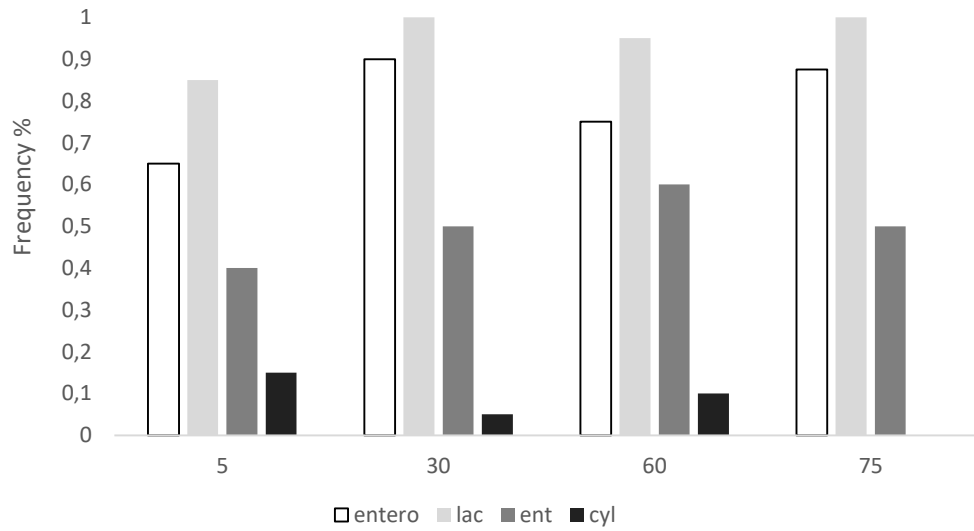
The digestibility data are shown in Table 3. The intake of DM (1097.8 g/d), CP (237.91 g/d), NDF(268.59 g/d), EE (185.51 g/d), OM (1044.02 g/d) and NFC (362.43 g/d), was greater ($P < 0.05$) during the second trial compared to first trial. Animals fed PROB had greater ($P < 0.05$) intakes of DM (1184.56 g/d) and CP (254.63 g/d) compared to EOPROB, and greater ($P < 0.05$) intake of NDF (320.36 g/d) compared to CO and EOPROB. The EE and OM intake was not affect ($P > 0.10$) by the treatments. The NFC intake was greater ($P < 0.05$) for animals fed EO (384.31 g/d) compared to CO (323.63 g/d), MON (323.84 g/d) and EOPROB (301.30 g/d). The NDF digestibility was greater ($P < 0.05$) for MON (924 g/d) compared to CO (786 g/d) and EOPROB (728 g/d). The DM, CP, EE, OM and NFC digestibility was not different ($P > 0.10$) among treatments.

Table 3. Intake and digestibility in two trials digestibility (1: 20-28 d; 2: 50-56 d) of dairy calves fed different additives in the concentrate: EO (Essential Oils), PROB (probiotic *Enterococcus faecium* NCIMB 10415), MON (monensina), EOPROB (EO + PROB), CON (Control).

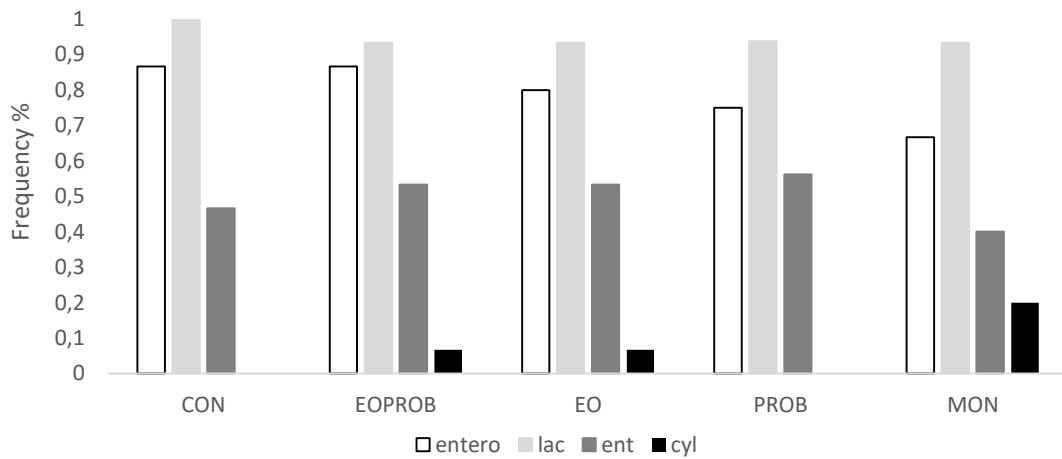
Item	Treatment					Trial		Standard Error	P value period	P value Treatment
	CON	MON	PROB	EO	EOPROB	1	2			
<i>g/d</i>	<i>Intake</i>									
DMI	839.67 ^{ab}	892.43 ^{ab}	1184.56 ^a	1042.63 ^{ab}	803.96 ^b	807.5 ^b	1097.8 ^a	118.08	0.001	0.022
CPI	193.03 ^{ab}	202.46 ^{ab}	254.63 ^a	226.6 ^{ab}	186.55 ^b	187.4 ^b	237.91 ^a	20.61	0.001	0.022
NDFI	124.57 ^b	182.29 ^{ab}	320.36 ^a	218.02 ^{ab}	121.9 ^b	118.27 ^b	268.59 ^a	62.7	0.001	0.030
EEI	180.62	180.26	181.85	182.11	173.25	173.7 ^b	185.51 ^a	3.95	0.001	0.571
OMI	825.73	965.30	1074.45	1011.05	767.91	813.75 ^b	1044.02 ^a	88.39	0.001	0.177
NFCI	323.63 ^{bc}	323.84 ^{bc}	362.0 ^{ab}	384.31 ^a	301.30 ^c	315.6 ^b	362.43 ^a	17.56	0.001	0.008
<i>g/g</i>	<i>Digestibility</i>									
DM	0.919	0.928	0.931	0.95	0.929	0.935	0.928	0.01	0.140	0.648
CP	0.908	0.905	0.918	0.936	0.934	0.927	0.913	0.01	0.141	0.627
NDF	0.786 ^{bc}	0.924 ^a	0.888 ^{abc}	0.903 ^{ab}	0.728 ^c	0.817	0.874	0.05	0.140	0.012
EE	0.969	0.977	0.972	0.978	0.950	0.967	0.969	0.01	0.140	0.377
OM	0.929	0.940	0.937	0.959	0.868	0.918	0.934	0.03	0.140	0.376
NFC	0.959	0.973	0.963	0.991	0.979	0.974	0.972	0.01	0.141	0.261

PCR analysis:

The PCR results (figure 2, table 4) show the relationship among the microorganisms and the time of feces sampling (5, 30, 60 and 75 days of life), and the relationship between the frequency of fecal bacteria and treatments. There was no interaction between period of collect and treatments ($P > 0.10$). On day 5 of life, before the beginning of the supplementation, it was possible to find the frequency of the 4 microorganisms analyzed, with a lower proportion of the probiotic strain. On day 30, the frequency of the microorganisms increased, except for the probiotic strain, which decreased. On day 60 the presence of Entero (*E. coli/ Hafnia/ Shigella*) and Lac (*Lactobacillus spp*) decreased slightly with respect to day 30, but Ent (*Enterococcus spp*) continued to increase, and the probiotic strain (Cyl) increased with respect to day 30. On day 75, when only control starter was fed to all animals, there was a small increase in the frequency of Entero and Lac, and a decrease in Ent, whereas Cyl was again not present. When the frequency of the different microorganisms was analyzed in each treatment (Figure 2b), the frequency of Lac was the same for all treatments with additives and higher for CON. In feces of animals fed EO and EOPROB it was observed exactly the same frequency of Ent and probiotic strain, the presence of Entero was only slightly smaller in EOPROB when compared to EO. In feces of animals fed PROB, it was observed that the frequency of Entero was lower compared to EOPROB and EO, and the presence of Ent was higher compared to other treatments. In stool samples from PROB and CON treatments, it was not possible to find the presence of the probiotic strain (*E. faecium* NCIMB10415). In feces of animals fed MON, it was observed a lower frequency of Entero and Ent compared to other treatments, and the frequency of the probiotic strain was greater in the MON treatment compared to other treatments.



a



b

Figure 2. Frequency of microorganisms (*E. coli*, *Hafnia*, *Shigella-Entero*, *Lactobacillus spp-Lac*, *Enterococcus spp-Ent*, and *Enterococcus faecium* NCIMB 10415-Cyl) in feces of dairy calves, fed different additives in the concentrate (b): EO (Essential Oils), PROB (probiotic *Enterococcus faecium* NCIMB 10415), MON (monensin), EOPROB (EO + PROB), CON (Control), and its interaction with days of life (5, 30, 60 and 75)(a).

Table 4. *P* values of PCR results of different microorganisms (*E. coli/Hafnia/shiguella*, *E. Faecium NCIMB10415*, *Enterococcus spp.*, and *Lactobacillus spp.*) present in feces of calves receiving different additives (Probiotic *E. faecium* NCIMB10415, Essential oils blend, Monensin, mixture of EO and PROB, and control).

<i>Bacteria</i>	<i>P</i> value	
	<i>Period*</i>	<i>Treatment**</i>
<i>E. coli/ Hafnia/ Shiguella (16S rRNA)</i>	0.2273	0.6473
<i>E. Faecium NCIMB10415 (plasmid)</i>	0.7923	0.7155
<i>Enterococcus spp. (16S rRNA)</i>	0.7957	0.999
<i>Lactobacillus spp. (16S rRNA)</i>	0.6643	0.9036

*period of collect: Moment that stool samples were collected.

**Treatment: Different additives supplied.

4. DISCUSSION

Intake and growth performance

The DMI increased for EO fed calves. Starter feed for suckling calves is commonly formulated with flavorizers to stimulate starter intake, and we suspect that EO might have a flavor effect on starter. Zeng et al.(2015) suggest that an increase in starter DMI when EO is supply in piglets, result in improved weight gain. Similar results were obtained in calves with increased ingestion of EO and mixtures of distillation residues compared to CON group (Li et al., 2012; Jeshari et al., 2016). However, Seifzadeh et al., (2017) found that the combination of EO and PROB for calves decreased intake compared to exclusively EO fed animals. On other hand, the combination of thymol and cinnamaldehyde would have the best potential to control the proliferation of pathogenic bacteria and contribute to better gut health (Li et al., 2012). Besides, carvacrol and thymol reduced the number of intra-epithelial lymphocytes and increased the ratio of villus height/crypt depth in the distal small intestine, suggesting also an improved gut health (Michiels et al., 2009). Therefore, improvement in gastro-intestinal health as a result of

EO supplementation may enhance the intestinal availability of essential nutrients for absorption, and consequently a better growth performance, thus greater feed intake (Jeshari et al., 2016). To ensure that it was a beneficial effect of EO, or a food flavor effect on starter concentrate is hard (Baser and Buchbauer, 2010)

At pre-weaning stage, the ADG and the FE were not different among treatments, these results coincide with other studies, where probiotics or EO were fed to calves before weaning (Cruywagen et al., 1996; Riddell et al., 2008; Chapman et al., 2017; Seifzadeh et al., 2017).

The significant increase in ADG and FE after weaning of EO-fed calves show that there was a positive residual effect of EO 15 days after stopping supplementation with that additive, and this response was also expressed in EOPROB fed calves. However, as we did not observe any significant PROB effect, it is likely that EOPROB-fed animals had an increased ADG and FE mainly due to EO effect. The EO are conformed of different chemical compounds, among which it is possible to find fatty acids as Oleic acid (18:1), linoleic acid (18:2), Palmitic acid (16:0), Stearic acid (18:0) and Linoleic acid (18:3) (Cakir, 2004; Taarit et al., 2010; Siddique et al., 2016). It is believed that these fatty acids have anti-inflammatory effects, reducing the stress commonly observed during 15-30 d of life, or during post-weaning period, since long-chain fatty acids activate the peroxisome proliferator-activated receptors (PPARs), which are members of the nuclear-hormone-receptor superfamily and transduce a wide variety of signals, including environmental, nutritional and inflammatory events; acting as a positive acute phase protein capable of decreasing the inflammatory response, and with long-term effects (Bionaz et al., 2013). This could explain why those animals that consumed EO before weaning, had better performance after weaning. In contrast, the MON and PROB group had a lower ADG and FE after weaning when compared to EO, and it was even lower

than before weaning, showing that there was no residual effect in those animals.

Fecal score

Before weaning, the FCI was lower for animals fed EO and MON additives, indicating lower incidence of diarrhea and greater intestinal health (Cho and Yoon, 2014). The mechanism of action of EO against enteropathogenic bacteria can be explained by its typical hydrophobicity, responsible for the disruption of bacterial structures that leads to increased permeability due to an inability to separate EO from the bacterial cell membrane (Nazzaro et al., 2013). The EO are generally most effective against Gram-positive microorganisms, since they manage to easily interact with the tetrapeptides presents in the membrane of peptidoglycans, inactivating enzymes such as transpeptidases, increasing permeability and destroying the cell (Juven et al., 1994; Lambert et al., 2001). While the Gram-negative bacteria are more resistant, due to lipopolysaccharides (consists of lipid A, the core polysaccharide, and the O-side chain) contained in their outer membrane (Nazzaro et al., 2013). However, Stein and Kil (2006) found that carvacrol, eugenol and thymol, are capable of disintegrating the outer membrane of Gram-negative bacteria such as *Escherichia coli* and *Salmonella* Typhimurium, two of these are compounds present in our EO blend (thymol, guaiacol, eugenol, vanillin, salicylaldehyde and limoneno). The activity of the EO and/or their components is not attributable to a single event (Helander et al., 1998; Ultee et al., 2002), because changes in molecular structures, like hydroxyl group (OH^-) can enhance antibacterial activity of some terpenes (Šarac et al., 2014). Considering the fact that five of the six (timol, guaiacol, eugenol, vanillin and salicylaldehyde) EO present in our blend contain the OH^- group, it could explain its high antimicrobial activity and the reduction of diarrhea in EO-fed calves.

On the other hand, monensin gets action against pathogenic bacteria, facilitating ion transport across the bacterian cytoplasmic membrane bacterian, by the formation of

liposoluble complexes with a hydrophobic exterior, and a hydrophilic interior able to bind cations as sodium and potassium, increasing the permeability of the cellular membranes to such ions, promoting an osmotic imbalance, increased energy expenditure and subsequent cell death (Russell and Strobel, 1989). This mechanism leads to improvements in animal performance and lower incidence of diarrhea.

The ability of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection, and several bile salt hydrolases (BSH) have been identified and characterized. BSH activity has been detected in *Enterococcus faecium* (Wijaya et al., 2004; Begley et al., 2006). However, BSH activity is considered a colonization factor favoring intestinal growth, as suggested by Moser and Savage. (2001), and it could be viewed as a potential virulence factor, especially in enterococcus strains that carry other recognized virulence traits (Wijaya et al., 2004). Many bacteria are able to deconjugate the bile salts by the specific hydrolasa, and this mechanism produce a reduction of cholesterol absorption at the intestinal level, increasing the cholesterol in the feces, and consequently greater passage rate (Kumar et al., 2012). However, there was no relationship between occurrence or severity of diarrhea and performance of PROB fed animals. Thus, the positive effect of probiotics on growth performance of calves may only be present when their health status is compromised (Timmerman et al., 2005).

The weekly data analysis of fecal consistency showed an increase in FCI from week 4 in the PROB-fed animals, and lower FCI for EO and MON fed animals. At day 35, all animals were dehorned, a very stressful procedure, but the results showed that animals fed EO and MON, were able to better face this moment without affecting their health, when compared to PROB-fed calves. After week 6, the fecal score for all animals increased significantly, which may have been due to the stress of the digestibility trial performed between weeks 6 and 7, and to post-weaning stress at week 8. After week 9

the fecal score started to decrease for all animals, indicating that animals adapted to the environment. Similar results were observed by Santos et al. (2015).

Digestibility

To date, no reasons have been found for the greater intake of fed PROB calves, but it is important to note that between weeks 4 and 6 these animals had a greater incidence of diarrhea and a greater intake could be a compensation mechanism due to the low nutritional and immunological status of the animals. Although, future studies should be developed to better understand this potential for compensating DMI of PROB-fed animal during severe stresses such as our digestibility trials.

Calves fed EO had a greater intake of NFC. The EO stimulate the secretion of the pancreatic amylase and increase its activity in the small intestine (Platel et al., 2002), which plays a major role in carbohydrate digestion and absorption. On the other hand, EO are capable of improving the digestibility of nutrients, since increase the secretions of saliva, bile and enhanced enzyme activity (Zeng et al., 2015), reduce protein degradation in the rumen by inhibiting the proliferation of bacteria capable of produce ammoniacal N or proteolytic, favoring the flow of these nutrients into the intestine, and, favoring the proteins digestion and absorption, besides to increase the activity of chymotrypsin (Platel et al., 2002; Duval and Hart, 2008). In addition, they may also enhance biliary secretion of bile acids, and stimulate the secretion of pancreatic lipase, which plays a major role in fat digestion and absorption (Platel et al., 2002). The EO are capable to decrease the number of pathogenic bacteria in the gut, improving the ability of epithelial cells to regenerate villi and thus improve intestinal absorption capacity (Zeng et al., 2015).

A greater digestibility of NDF was expected in MON fed animals, since the

ionophores supplementation increases the ruminal concentration of propionate (Quigley et al., 1992; Schären et al., 2017), which in turn causes a decrease in consumption, stimulating the sense of satiety (Oba and Allen, 2003; Chambers et al., 2015), and consequently a greater retention rate of feed in the rumen (Lemenager et al., 1978). Then, the increase in the digestibility of the fiber can be a result of the longer retention time of the fiber in the rumen that favors the microbial digestion. Nevertheless, recent studies did not find differences in VFA concentrations or rumen pH and the ruminal rate when EO or MON were supplied (Newbold et al., 2004; Castillejos et al., 2007; Meyer et al., 2009; Ishlak et al., 2015). Therefore, this discrepancy among the different studies may be related to several factors such as diet composition, the period of adaptation to the product, the time of sample collection and the type and concentration of the feed additives (Meyer et al., 2009; Ishlak et al., 2015).

Performed at 50-56th days of life was already expected as the animals were growing and the rumen and intestine digestion capacity increase up to 70% around the 56th day of life (Baldwin et al., 2004).

Fecal microorganisms

It was not possible to find the presence of probiotic PROB, in those animals fed the same probiotic, nor in those of the control treatment. This result indicates that possibly the probiotic was not able to survive in the gastrointestinal tract of the calves. In order to survive in the gastrointestinal tract, bacteria need to adhere to the intestinal wall (or develop faster than the speed of peristalsis), and to reach and colonize the intestine, which requires bacteria to be resistant to acid pH and bile acids (Gismondo et al., 1999). In PROB animals had a greater FCI, with a greater incidence of diarrhea and no effect on nutrient digestibility, corroborates with the hypothesis that possibly the probiotic bacteria

did not survive and therefore there was no positive effect for the animals in this treatment.

In animals fed MON, EO and EOPROB, it was possible to find the presence of the probiotic bacteria (*Enterococcus faecium* NCIMB 10415-Cyl) (figure 2b), an effect that could be explained by the ability of MON and EO to increase the growth of beneficial bacteria. Since, the bacteria *E. faecium* is naturally present in the gastrointestinal tract of calves, being found mainly in the saliva, and small intestine (Schneider et al., 2004), it could be suggested that these treatments managed to promote the growth of this bacteria, specifically the strain studied in this study. This also explains why the probiotic strain did not appear in the feces collected in the day 75, when the calves did not receive the additives.

Finally, the PROB treatment was similar to the CON treatment, with the absence of the probiotic bacteria, while in contrast the EO treatment showed a similar result to the EOPROB treatment, reinforcing the hypothesis that the probiotic was not present in the gastrointestinal tract of those animals. However, the presence of the other bacteria analyzed were not affected by any of the treatments.

5. CONCLUSION

The EO proved to be a good alternative to improve the health of calves as it decreased the incidence of diarrhea. In addition, the essential oils allowed a greater consumption of dry matter, improved digestibility of the nutrients and allows residual effects after weaning with greater ADG and FE. The monensin improved health of calves, decreasing the incidence of diarrheas in pre-weaning period.

E. faecium NCIMB 10415 probiotic did not present positive results in the evaluation of fecal score, as it did not appear to survive in the gastrointestinal tract of the calves. Besides, ADG and nutrient digestibility were not affected by PROB.

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