

## Prophylactic use of an ultra-diluted complex on polymorphonuclear leukocyte function and respiratory scores of weaned Holstein calves immediately after grouping

Uso profilático de um complexo ultra-diluído na função de leucócitos polimorfonucleares e escores respiratórios de bezerros holandeses desmamados imediatamente após o agrupamento

Uso profilático de un complejo ultradiluido sobre la función de los leucocitos polimorfonucleares y las puntuaciones respiratorias de los terneros Holstein destetados inmediatamente después de la agrupación

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### Abstract

The aim of the study was to evaluate the effect of an ultra-diluted complex supplementation as a prophylactic strategy on immunity, performance, and respiratory scores of weaned Holstein calves immediately after grouping. Thirty-six weaned Holstein female calves (80.4±1.3 days old; 105.6±10.4 kg) were allocated to 6 pens (n=6 per pen) in a completely randomized design experiment in a double-blind, placebo-controlled trial. During a 28 days period, animals received a total mixed ration and were enrolled into two different groups (n=18 per group): 1) Control (basal diet + calcium carbonate, top-dressed at 30 g/animal/d – ultra-diluted placebo vehicle), or 2) ultra-diluted complex (basal diet + TopVita™-Real H, top-dressed at 30 g/animal/d – *Sulphur*:10<sup>-60</sup> + *Viola tricolor*:10<sup>-14</sup> + *Caladium seguinum*:10<sup>-30</sup> + *Zincum oxydatum*:10<sup>-30</sup> + *Phosphorus*:10<sup>-60</sup> + *Cardus marianus*:10<sup>-60</sup> + *Colibacillum*:10<sup>-30</sup> + *Podophyllum*:10<sup>-30</sup> + Vehicle: calcium carbonate; q.s. 1kg). Blood samples were collected from each heifer at enrollment and 28 days later to assess polymorphonuclear leukocyte (PMNL) function and blood cell count. Body weight was assessed at enrollment and 28 days later at the end of the study. Regarding respiratory-screening process, a calf scoring system modified for calves in group pens was used. There was no effect of prophylactic ultra-diluted treatment on PMNL, nor it affected lymphocytes count and its ratio. Besides, the ultra-diluted product did not affect body weight and ADG. Further, no effect was observed in respiratory scores throughout the study period. In conclusion, the ultra-diluted complex did not improve blood cells count and PMNL function, nor it had impact on the performance of weaned Holstein calves after grouping.

**Keywords:** Calves; Homeopathy; Oxidative burst; Phagocytosis; Pneumonia.

### Resumo

O objetivo do estudo foi avaliar o efeito de uma suplementação de complexo ultra-diluído como estratégia profilática sobre a imunidade, desempenho e os escores respiratórios de bezerros Holandeses desmamados imediatamente após o agrupamento. Trinta e seis bezerras holandesas desmamadas (80,4 ± 1,3 dias de idade; 105,6 ± 10,4 kg) foram alocadas em 6 baias (n = 6 por baia) em um experimento de desenho completamente randomizado em um ensaio duplo-cego controlado por placebo. Durante um período de 28 dias, os animais receberam uma ração mista total e foram incluídos em dois grupos diferentes (n = 18 por grupo): 1) Controle (dieta basal + carbonato de cálcio, coberto com 30 g / animal

/ d - ultra- veículo placebo diluído), ou 2) complexo ultra-diluído (dieta basal + TopVita™ -Real H, coberto com 30 g / animal / d - Enxofre: 10-60 + Viola tricolor: 10-14 + Caladium seguinum: 10 -30 + Zinco oxidado: 10-30 + Fósforo: 10-60 + Cardus marianus: 10-60 + Colibacilina: 10-30 + Podophyllum: 10-30 + Veículo: carbonato de cálcio; qsp. 1 kg). Amostras de sangue foram coletadas de cada novilha no momento da inscrição e 28 dias depois para avaliar a função dos leucócitos polimorfonucleares (LPMN) e contagem de células sanguíneas. O peso corporal foi avaliado no momento da inscrição e 28 dias depois no final do estudo. Com relação ao processo de triagem respiratória sistemática, foi utilizado um sistema de pontuação de bezerros modificado para bezerros em baias coletivas. Não houve efeito do tratamento profilático ultra-diluído sobre os LPMN, tampouco afetou a contagem de linfócitos e sua proporção. Além disso, o produto ultra-diluído não afetou o peso corporal e o GMD. Além disso, nenhum efeito foi observado nas pontuações respiratórias ao longo do período de estudo. Em conclusão, o complexo ultra-diluído não melhorou a contagem de células sanguíneas e a função dos LPMN, nem teve impacto no desempenho de bezerros Holandeses desmamados após o agrupamento.

**Palavras-chave:** Bezerros; Fagocitose; Homeopatia; Pneumonia; Queima oxidativa.

### Resumen

El objetivo del estudio fue evaluar el efecto de un suplemento complejo ultra-diluido como estrategia profiláctica sobre la inmunidad, el rendimiento y las puntuaciones respiratorias de los terneros holandeses destetados inmediatamente después del agrupamiento. Treinta y seis novillas Holstein destetadas ( $80,4 \pm 1,3$  días de edad;  $105,6 \pm 10,4$  kg) se alojaron en 6 corrales ( $n = 6$  por corral) en un experimento de diseño completamente aleatorizado en un ensayo doble, ciego controlado con placebo. Durante un período de 28 días, los animales recibieron una ración total mixta y fueron incluidos en dos grupos diferentes ( $n = 18$  por grupo): 1) Control (dieta basal + carbonato cálcico, rematado con 30 g / animal / d - ultra - vehículo placebo diluido), o 2) complejo ultradiluido (dieta basal + TopVita™ -Real H, cubierto con 30 g / animal / d - Azufre: 10-60 + Viola tricolor: 10-14 + Caladium siguiente: 10-30 + Zinc oxidado: 10-30 + Fósforo: 10-60 + Cardus marianus: 10-60 + Colibacilina: 10-30 + Podophyllum: 10-30 + Vehículo: carbonato de calcio; cs 1 kg). Se recolectaron muestras de sangre de cada novilla en el momento de la inscripción y 28 días después para evaluar la función de los leucocitos polimorfonucleares (LPMN) y los recuentos de células sanguíneas. El peso corporal se evaluó en el momento de la inscripción y 28 días después al final del estudio. En cuanto al proceso de cribado respiratorio sistemático, se utilizó un sistema de puntuación de terneros modificado para terneros en corrales grupales. No hubo ningún efecto del tratamiento profilático ultradiluido sobre los LPMN, ni afectó el recuento de linfocitos y su proporción. Además, el producto ultradiluido no afectó al peso corporal ni a la ganancia diaria promedio. Además, no se observó ningún efecto sobre las puntuaciones respiratorias durante el período de estudio. En conclusión, el complejo ultradiluido no mejoró el recuento de células sanguíneas ni la función del LPMN, ni afectó el rendimiento de los terneros holandeses destetados después del agrupamiento.

**Palabras clave:** Terneros; Fagocitosis; Homeopatía; Neumonía; Quemadura oxidativa.

## 1. Introduction

Despite of diet changes, individual milk-fed calves usually experience a new environment and social interaction after weaning process. The weaning management has been made in order to increase labor efficiency, since fewer workers are needed to monitor the animals. The new housing systems follow weaning have several novel items such as feeding and drinking equipment, which may become this period a stressful phase for animals since this is the first time, they experience real social contact with other mates. Studies have identified the benefit of group pen systems before weaning, either similar or older mate, because it may decrease the stress caused by this regrouping strategy (Bolt et al., 2017; De Paula Vieira et al., 2012). Finally, milk-feeding rearing method, which has been wide used in dairy farms, may impact stress levels during the process of regrouping after weaning management (Bolt et al., 2017).

Stress has been associated with immune system failure, since several hormones such as cortisol and catecholamine have been linked to an inefficient immune system functionality (Alhussien & Dang, 2020). Hulbert and Ballou (2012) detected negative impact of grouping strategy on immune functionality of weaned dairy calves, even though they grouped 3 animals each pen in comparison with individual housing weaned animals. Indeed, respiratory disease was the single largest cause of weaned heifer deaths in the U.S. (NAHMS, 2007). Thus, alternatives need to be studied in order to decrease respiratory disease incidence during this phase.

Ultra-diluted remedy was conceived in 1796 by Samuel Hahnemann. It is a diluted form of a substance that causes symptoms of a disease in healthy people when in high doses. However, in the diluted form, it would heal similar symptoms in

sick people (Hahnemann, 1810 translated by Kunzli et al., 1982). The early concepts consist in treat the specific symptoms of a single individual; however, in a herd perspective, the concept of homotoxicological remedies has been suggest. Homotoxicological remedies, herein used, use a combination of several ultra-diluted medicine that may be prescribed based on a disease pattern for a set of individuals, favoring its use by veterinarians around the world (Arlt et al., 2009). Although, the ultra-diluted technology is widespread among the dairy producers, more well-designed studies must be assessed to confirm its reliability (Doehring & Sundrum, 2016). The aim of this study was to evaluate the immune function, performance, and respiratory scores of weaned dairy calves immediately after grouping.

## 2. Methodology

The methodologic approach herein used may be classified as a quantitative method. A quantitative or numerical data is collected due to the use of measurements of quantities, which is obtained through metrology (numbers with their respective units; Yin, 2015). All procedures performed in this study were approved by the Animal Care and Use Committee (IACUC, protocol #20018-02). Based on previous blood samples analysis from the studied herd, baseline neutrophils count in weaned calves housed in superhutches was anticipated to average  $4 \times 10^6$  cells/mL and standard deviation =  $1 \times 10^6$  cells/mL, with an assumption that ultra-diluted treatment would reduce neutrophil count by at least 25% ( $1 \times 10^6$  cells/mL unit). To detect this reduction, 17 calves per treatment for each group was required to ensure a power ( $1 - \beta$  error probability) of 0.80. To ensure this number of animals per treatment, herein we enrolled 18 animals each group.

Between February 2020 and March 2020, 36 clinically healthy weaned Holstein calves were randomly selected at a commercial dairy located in west Texas, USA. The dairy milked 3700 Holstein cows three times daily in a 70-stall rotary milking parlor. After being weaned about  $60 \pm 1$  d of age, the calves remained at hutches until  $80.4 \pm 1$  d of age, when the calves were moved to the group pen. The calves were housed in outdoor, plastic superhutches with an attached pen. Superhutches, is a large calf hutch ( $6 \times 3$  m) that provides transitional housing for a small group of 6 weaned calves per group. A total of 6 superhutches (6 animals each) were enrolled for this study. The superhutches were bedded with cottonseed hulls and equipped with a feed bunk that provided space for 6 calves and 1 water bowl. When moved into groups, the calves continued to receive the same starter fed in the milk-feeding period. The animals had *ad libitum* access to water and starter. The experimental period lasted 28 days after enrollment date.

The weaned growing calves were allocated into 2 treatments at the day of grouping (3 superhutches/group and  $n = 18$  animals/group): (1) Control: untreated controls (basal diet + calcium carbonate, top-dressed at 30g/animal/d – placebo); or (2) Ultra-diluted complex : treated with a ultra-diluted complex (basal diet + TopVita™ - Real H, top-dressed at 30 g/animal/d - Sulphur:  $10^{-60}$  + *Viola tricolor*:  $10^{-14}$  + *Caladium seguinum*:  $10^{-30}$  + *Zincum oxydatum*:  $10^{-30}$  + *Phosphorus*:  $10^{-60}$  + *Cardus marianus*:  $10^{-60}$  + *Colibacillinum*:  $10^{-30}$  + *Podophyllum*:  $10^{-30}$  + Vehicle: q.s. 1kg – calcium carbonate; TopVita™ – Real H Company, Campo Grande, MS, Brazil).

Blood samples were collected from each heifer at enrollment and 28 days later, from the jugular vein using a Vacutainer tube with lithium heparin, a Vacutainer tube with potassium EDTA, and a 20-gauge x 2.54-cm Vacutainer needle (Becton, Dickinson and Company, Franklin Lakes, NJ). After collection, the heparinized blood was stored in an ice chest without any ice to preserve their phagocytic and oxidative burst capacity (Sellers et al., 2013). The EDTA and coagulated blood samples were transported to the laboratory on ice. The heparinized and EDTA blood samples were processed in the same day of collection for measures of hematology and *ex vivo* PMNL responses.

Leukocyte count and differentials were performed using a hematology analyzer (IDEXX Procyte DX, Westbrook, ME), and the variables of interest were neutrophil count and neutrophils to lymphocytes ratio. Flow cytometry was used to determine the phagocytosis and oxidative burst capacity of peripheral PMNL and the quantification of the adhesion molecule L-selectin

(CD62L), with minor modifications (L. E. Hulbert et al., 2011). To measure the phagocytic and oxidative burst capacity, 100  $\mu\text{L}$  of heparinized whole blood were incubated for 15 min in an ice bath. After this initial incubation, 20  $\mu\text{L}$  of a 100  $\mu\text{M}$  solution of dihydrorhodamine (Invitrogen, Carlsbad, CA), and 20  $\mu\text{L}$  of a  $10^9$  cfu/mL propidium iodine labeled *E. coli* suspension were added to the blood, and then incubated in a 38.5°C water bath for 10 min (negative controls were incubated in an ice bath for 10 min). Then, samples were immediately placed on an ice bath for 5 min, and erythrocytes were hypotonically lysed, and washed with PBS. Dual-color flow cytometry was performed using an Attune flow cytometer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA). The PMN population was gated using forward and side scatter plots. The mean fluorescence intensity (MFI) and proportion of PMNs that performed phagocytosis and oxidative burst were acquired using the optical filters BL3 (excited by a 488 nm laser on a 695/40 filter) and BL1 (excited by a 488 nm laser on a 530/30 filter), respectively. Negative controls were used to determine negative and positive signals on the BL1 by BL3 scatterplot used to assess PMNs that performed phagocytosis and oxidative burst. Phagocytosis and oxidative burst indexes were created by multiplying the proportion of responding PMNL by the corresponding MFI: [index = (positive %)  $\times$  (MFI)], as greater index values indicate greater phagocytic and oxidative burst activities (McCarthy et al., 2016). To determine the expression of the adhesion molecule L-selectin, 50  $\mu\text{L}$  of EDTA blood samples were mixed with 50  $\mu\text{L}$  of PBS containing 1  $\mu\text{g}$  of a monoclonal antibody mouse IgG1-isotype (catalog number: BOV2046, clone: BAQ92A; Veterinary Microbiology and Pathology Monoclonal Antibody Center, Pullman, WA). After a 1 h incubation in an ice bath, erythrocytes were hypotonically lysed. After centrifugation, the leukocyte pellet was resuspended in a 50  $\mu\text{L}$  solution of fluorescein-labeled secondary antibody at a 1:400 dilution (F(ab')<sub>2</sub> anti-mouse IgG:FITC; AbD Serotec Raleigh, NC) and incubated for 1 h in an ice bath. After a PBS wash, samples were analyzed using single-color flow cytometry. The PMN population was gated as previously described, and the MFI for L-selectin was gathered using BL-1. Data were analyzed using Attune Cytometric software (Life Technologies, Thermo Fisher Scientific Inc.).

Body weight was assessed at enrollment and 28 days later at the end of study. Through the practical and reliable technique, body weight was estimated using calibrated weight tapes measuring heart girth circumference (Heinrichs et al., 1992). These measurements were used to calculate the body weight average daily gain (ADG) during study period (final body weight – initial body weight / 28 days).

To respiratory-screening process, a calf scoring system modified for calves in group pens was used according to McGuirk and Peek (2014). This group pen respiratory scorer is available at <https://www.vetmed.wisc.edu/fapm/svm-dairy-apps/group-pen-respiratory-scorer/>. The categorization was made scoring nasal discharge, ocular discharge, ear tilt, and coughing. This modified scoring system identify diseases of the group instead of individual animals. If the group/pen is classified into having more than 25% of the animals with abnormal in 2 or more categories scored, this group would be classified as a high risk to develop respiratory disease. Abnormal scores are stated as score 2 or 3 from 0 to 3-point scale. This tool is used in farm's calf health-screening program to obtain early diagnostic of respiratory diseases in calves (McGuirk & Peek, 2014).

The effect of ultra-diluted treatment on PMNL function, cells count, body weight, and growth (ADG) was performed using the MIXED procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). The model included the fixed effects of treatment and random effect of pen. The random effect of pen was used to recognize the experimental unit for treatment group. In addition, repeated observations within a pen were modeled using a compound symmetry residual variance-covariance structure. Respiratory scores were modeled using a categorical multinomial distribution fitted with a cumulative logit link function in GLIMMIX procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). Least square means and proportions are reported for all parameters evaluated. For all the analyses, differences detected at  $P \leq 0.05$  were considered significant.

### 3. Results and Discussion

Several management changes are imposed to the animals over the heifer-raising period. The most cited changes reported are introduction to a new feeding and housing system, regrouping, social isolation, and diet modifications. These changes require extensive behavioral resiliency of the animals to acclimate to them (De Paula Vieira et al., 2012). A study about a profile of calves' management practices in the U.S. related that about 80% of farms move calves into group pens around 8 weeks of age. Further, large dairies have moved their calves to group pens later than small operations in order to decrease the spread of pathogenic microorganisms in milk-feeding period (NAHMS, 2007). In an excellent review, Bøe and Færevik (2003) reported that grouping of unfamiliar animals increases aggression, social stress, and locomotor behavior in calves. Additionally, this practice may negatively impact feed intake, body weight ADG, and immune functionality of dairy calves after weaning (Lindsey E. Hulbert & Ballou, 2012).

We did not detect any effect of ultra-diluted supplementation on immune function and blood cells count of weaned dairy calves after grouping (Table 1). Additionally, even though any effect was detected on BW ADG, satisfactory gains was detected between the groups (1.0 and 0.99 kg/d for controlled and ultra-diluted treated group, respectively). Differently of Hulbert and Ballou (2012), our findings may be explained through the absence of stress effects on neutrophil functionality at this lifetime of the calves, similarly as mentioned by Leblanc (2020), who reviewed the immune function of transition dairy cows under social stress. This author reported that the association between stress (acute or chronic) and impaired immune function has not been confirmed in controlled trials, concluding that competitive displacement at the feed bunk in overcrowded groups may have no effect alone on immune function (phagocytosis and oxidative burst) of transition dairy cows. To date, there is no well-designed studies verifying the effects of ultra-diluted products on immune function of weaned dairy calves to perform relevant discussion among ultra-diluted strategies.

**Table 1.** Blood measurements of weaned Holstein calves supplemented with ultra-diluted complex (n=18) or control (n=18) adjusted for pen.

Item	Treatment		SEM	P-value
	Control	Ultra-diluted		
Phagocytosis, %	74.6	76.7	3.92	0.72
Phagocytosis, MFI	599.2	601.1	26.2	0.96
Phagocytosis index	449.5	467.8	40.7	0.77
Oxidative burst, %	56.4	57.2	4.76	0.91
Oxidative burst, MFI	1067.7	1153.2	65.3	0.41
Oxidative burst index	611.1	680.5	81.1	0.58
L-selectin, MFI	386.3	437.8	26.2	0.24
Neutrophil count, cells x 10 <sup>6</sup> /μL	3.8	4.1	0.61	0.62
Lymphocyte count, cells x 10 <sup>6</sup> /μL	6.2	6.1	0.44	0.93
Neutrophil : Lymphocyte	0.64	0.67	0.24	0.78

Source: Authors.

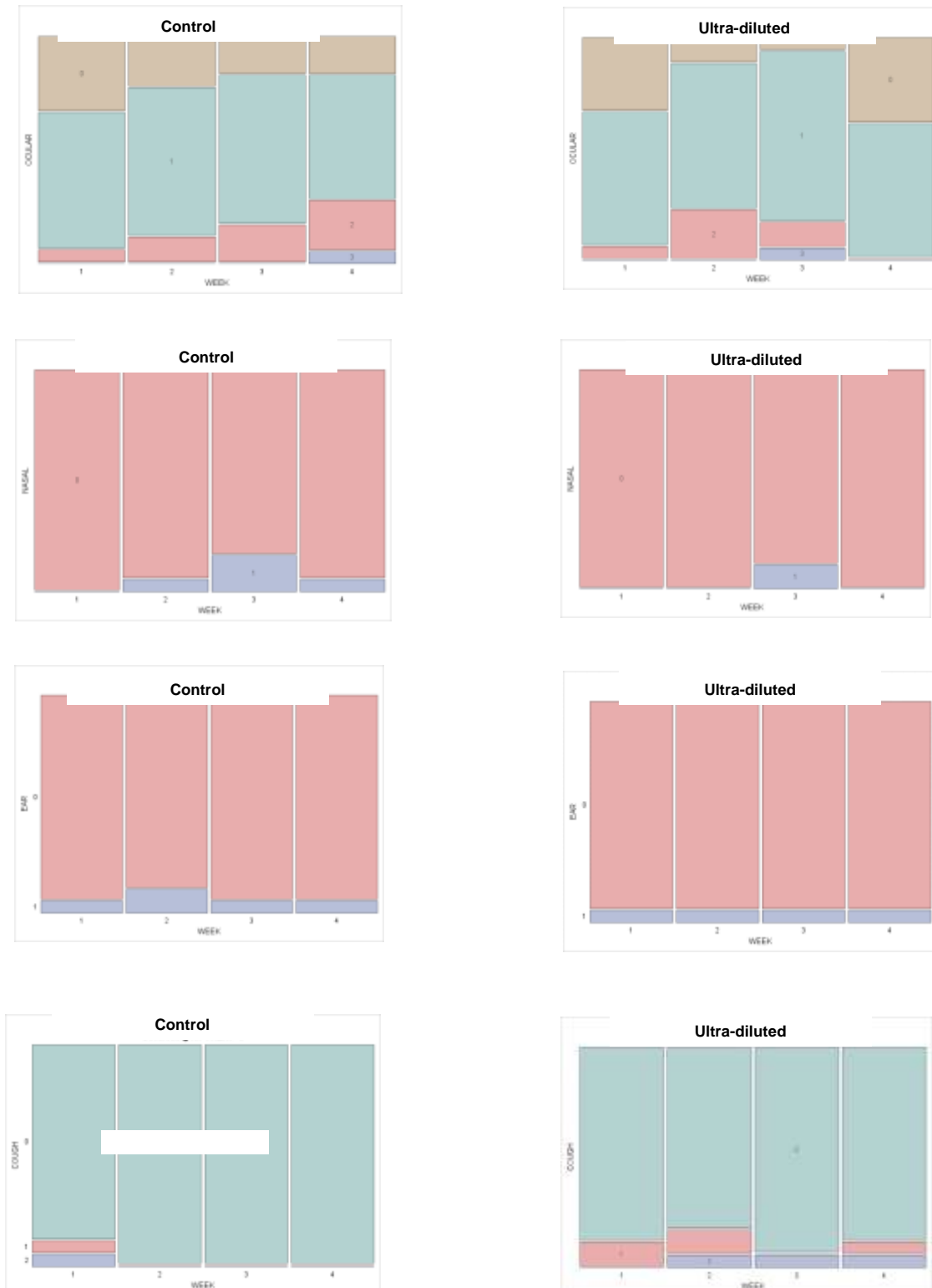
**Table 2.** Body weight (BW) and BW average daily gain (ADG) adjusted for pen of weaned Holstein calves supplemented with ultra-diluted complex (n=18) or control (n=18).

Item	Treatment		SEM	P-value
	Control	Ultra-diluted		
Initial BW, kg	105.1	106.1	2.56	0.78
Final BW, kg	130.8	130.3	2.94	0.89
BW ADG, kg/d	1.0	0.99	0.12	0.91

Source: Authors.

Respiratory disease is the most diagnosed illness in weaned dairy calves. About 93.3% of respiratory disease diagnosed in U.S. dairies were treated with an antibiotic in a survey published in 2007 (NAHMS, 2007). Additionally, respiratory disease was the single largest cause of weaned heifer deaths (46.5%). Lundborg et al. (2005) and Svensson and Liberg (2006) studies indicate that size of the group after commingling matters, whereas calves housed in large group pens had higher risk for respiratory disease. In this study, prophylactic supplementation of an ultra-diluted complex did not alter the respiratory scores in weaned dairy calves after grouping (Figure 1). As mentioned earlier, to date, there is no randomized, double-blind, placebo-controlled trial reporting the effect of an ultra-diluted medicine on heifer health. Additionally, low quality studies have been reported for livestock in some literature reviews both in animals and humans (Doehring & Sundrum, 2016; Mathie & Clausen, 2015).

**Figure 1.** Estimated cumulative probabilities of respiratory scores (ocular, nasal, ear, and cough parameters) in weaned Holstein calves during 4 weeks after grouping. Animals were receiving either ultra-diluted or control treatments. Respiratory scores were modeled using a categorical multinomial distribution fitted with a cumulative logit link function. No difference was detected between the groups among the different score parameters (Treatment:  $P > 0.05$ ; Week:  $P > 0.05$ ; and Treatment x Week:  $P > 0.05$ ).



Source: Authors.

## 5. Conclusion

In conclusion, prophylactic use of an ultra-diluted complex did not alter PMNL function, growth, nor it had effect on respiratory score of weaned growing calves during 28 d immediately after grouping.

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