Veterinary Parasitology 269 (2019) 57–64

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Long-term use of yeast fermentation products in comparison to halofuginone for the control of cryptosporidiosis in neonatal calves

Juan Vélez[a], Malin K. Lange[a], Peter Zieger[b], Ilkyu Yoon[b], Klaus Failing[c], Christian Bauer[a,*]

[a] Institute of Parasitology, Justus Liebig University Giessen, Schubertstrasse 81, 35392, Giessen, Germany
[b] Diamond V, Cedar Rapids, IA, 52404, USA
[c] Unit of Biomathematics and Data Processing, Faculty of Veterinary Medicine, Justus Liebig University, Giessen, Germany

ARTICLE INFO

Keywords:
Cryptosporidium parvum
Calf
Neonatal diarrhea
Saccharomyces cerevisiae
Fermented product
Halofuginone

ABSTRACT

The objective of this study was to compare the effect of non-GMO *Saccharomyces cerevisiae* fermentation products (SCFP) with that of a halofuginone treatment against *Cryptosporidium parvum* infection in pre-weaned calves on a commercial dairy farm. A total of 123 neonatal female calves, housed in individual hutches, were enrolled sequentially based on date of birth in 41 blocks of 3 animals each. Calves within each block were allocated to one of 3 treatments: remaining untreated, fed with SCFP (Diamond V SmartCare® at 1 g/d in milk and NutriTek® at 5 g/d in starter grain) for the first 63 days of life, or treated with halofuginone (0.1 mg/kg/d) for the first 7 days of life. Fecal samples collected on days 4–21 post-partum were examined for both *Cryptosporidium* oocysts and coproantigen. The presence and intensity of diarrhea were monitored by scoring daily for the first 4 weeks of life. Calves were weighed at 0, 21, 42 and 63 days of age. Almost all calves were *Cryptosporidium*-positive at least once during the study. Halofuginone significantly reduced the number of *Cryptosporidium*-positive fecal samples as compared to the two other groups. Based on the coproantigen scores, both halofuginone and SCFP feeding significantly reduced the intensity of *Cryptosporidium* infection as compared to the untreated group. Diarrhea was recorded in almost all calves at least once. Neither the proportion of diarrheic calves nor the intensity and duration of diarrhea differed among the 3 treatment groups significantly. The mean daily weight gain during the first 3 weeks of life was significantly lower in halofuginone treated calves than in both other groups; however, at the end of the study period the total weight gain did not significantly differ among the 3 treatment groups. In conclusion, the clinical results and weight gains of pre-weaning supplementation with the SCFP were neither better nor worse than the 7-day halofuginone treatment suggesting that the SCFP feeding may be from the clinical point of view a natural alternative measure, instead of halofuginone treatment, in bovine cryptosporidiosis.

1. Introduction

Cryptosporidium species are minute protozoan pathogens of vertebrates (mammals including man, birds, reptiles, fish and amphibians) with a worldwide occurrence (Fayer, 2008; Deplazes et al., 2016). They belong to the phylum Apicomplexa and have been recently reclassified from coccidian to gregarine parasites (Cavalier-Smith, 2014; Thompson et al., 2016). More than 30 *Cryptosporidium* spp. are known at present, the genotype B ('bovine type') of the *C. parvum* complex being a major enteropathogen in neonatal calves (Broglia et al., 2008; Feng et al., 2018; Holzhausen et al., 2019). *Cryptosporidium* spp. have a direct life cycle. In brief, infected hosts shed infectious oocysts with their feces, and new hosts become infected by oral ingestion of oocysts as host-to-host route, from their environment or via oocyst-contaminated drinking water or food. In mammals, *Cryptosporidium* spp. primarily infect the intestinal epithelium parasitizing the microvillous brush border of enterocytes. The endogenous life cycle includes a first asexual multiplication (merogony) followed by a sexual development (gamogony) and a second asexual multiplication (sporogony) resulting in huge quantities of infectious oocysts which are shed with feces. Oocysts can remain viable and infectious in the environment for months (Fayer, 2008; Deplazes et al., 2016).

Cryptosporidium infections are very common and endemic in many cattle farms worldwide, especially in large dairy enterprises (Joachim et al., 2003; Trotz-Williams et al., 2007; Al Mawly et al., 2015; Delafosse et al., 2015; Niine et al., 2018; Urie et al., 2018a, b). *C. parvum* is one of the four main causes of diarrhea in calves within the first 3 weeks of life (Gillhuber et al., 2014; Al Mawly et al., 2015;

* Corresponding author.
E-mail address: christian.bauer@vetmed.uni-giessen.de (C. Bauer).

https://doi.org/10.1016/j.vetpar.2019.04.008
Received 27 February 2019; Received in revised form 4 April 2019; Accepted 18 April 2019

Anonymous, 2017). Calves become infected within the first day(s) of life. The infection may cause no clinical symptoms, but is often responsible for a watery diarrhea with acute onset, which can be accompanied by depression, weakness and anorexia. The disease results from a severe villous atrophy in the small intestines. The *Cryptosporidium*-caused diarrhea generally lasts several days and is self-limiting because of the development of immunity (Thomson et al., 2017).

Cryptosporidium spp. are more or less completely insensitive to usual anticoccidial drugs, which are effective against *Eimeria* spp. and other coccidian parasites in animals (Stockdale et al., 2008; Shahiduzzaman and Daugschies, 2012), possibly because of their gregarine character. There are two compounds used in the field that were found to have an anticryptosporidial effect in calves after oral administration for several days (treatment starting from the first day of life): halofuginone (Silverlås et al., 2009; De Waele et al., 2010; Trotz-Williams et al., 2011; Almawly et al., 2013; Niine et al., 2018) and paromomycin (Fayer and Ellis, 1993; Grinberg et al., 2002). Halofuginone is the only drug approved against *Cryptosporidium* for use in neonatal calves by the authorities in Europe (Anonymous, 2000). However, in a meta-analysis of results from 14 respective studies, it was concluded that "some beneficial effects were found for the use of halofuginone as prophylactic treatment against cryptosporidiosis, but the substance is toxic and should be used only with severe *C. parvum*-associated diarrhoeal problems" (Silverlås et al., 2009). In recent studies halofuginone given prophylactically resulted in reduced oocyst shedding, but did not improve clinical symptoms or bodyweight gain of calves as compared to untreated controls (Trotz-Williams et al., 2011; Almawly et al., 2013), or was even negatively associated with the weight gain (Niine et al., 2018).

It was reported recently that 4-week feeding of neonatal *Cryptosporidium*-infected calves with *Saccharomyces cerevisiae* fermentation products resulted in significantly less fragmented and atrophied villi of the lower small intestines in comparison to untreated controls, suggesting a preventive effect of these products against the infection (Vázquez Flores et al., 2016). Therefore, the present longitudinal (cohort) study was performed to determine the parasitological, clinical and economical effects of *S. cerevisiae* fermentation products against *Cryptosporidium* infections in neonatal calves in comparison with both a 'positive' (halofuginone treated) and 'negative' (untreated) control group.

2. Materials and methods

2.1. Study location and time

The study was performed on a commercial dairy cow farm with approximately 1600 cows in eastern Germany from December 2017 to April 2018. The farm was known to harbor *Cryptosporidium* infection endemically, and halofuginone had been prophylactically used in the past.

2.2. Study calves

A total of 123 female calves, all of Holstein Frisian breed and being healthy on the day of birth (respective study day 0), was enrolled in the study. Feeding and management practices were followed by the routine procedures of the farm. Calves were immediately separated from their dams after birth, identified by numbered ear tags and housed outdoor in individual hutches on concrete floor supplied with straw bedding for the first 9 weeks of life. Each calf was given approximately 4 l colostrum from its dam within the first 3 h after birth. Thereafter, they were fed with whole waste milk twice a day and, from the 2nd week of life, additional grain was offered for *ad libitum* consumption. The calves were from 56 primiparous, 23 secundiparous and 44 multiparous cows (25, 10, 6 and 3 cows with 3, 4, 5 or 6 lactations, respectively). Secundi- and multiparous dams had been vaccinated against Rotavirus and Coronavirus infection before birth of their calves, but primiparous dams did not get this vaccination following the farm practices.

2.3. Test materials

Two *S.cerevisiae* fermentation-based non-GMO feed additives (SCFP), SmartCare® and NutriTek®, were supplied by Diamond V. Both products were stored at room temperature and given according to the manufacturer's instructions: 1 g SmartCare/calf/day was mixed in milk and given orally, and 5 g NutriTek/calf/day were mixed with a small amount of starter feed and offered directly for feeding, both at the same time each day on days 1–63.

Halofuginone (HALO; Halocur® 0.5 mg/ml, MSD Tiergesundheit, Germany) was given according to the manufacturer's instructions: calves of 35–45 kg and 45–60 kg birth weight received 8 ml and 12 ml Halocur, respectively; lighter or heavier calves received 2 ml Halocur/10 kg (corresponding to a dose rate of 0.1 mg halofuginone/kg). The drug was orally administered using the product´s dispenser once daily after morning feeding on days 1–7.

2.4. Treatment groups

The study calves were sequentially enrolled in 41 blocks of 3 animals based on their date of birth, and calves within each block were allocated to one of 3 treatments: 1) remaining untreated (CON); 2) fed with SCFP; or 3) treated with HALO. After allocation any calf suffering from a disease other than diarrhea during its first 3 weeks of life as well as both calves in the same block were removed from the study and replaced by a new block of 3 calves.

2.5. Sampling and clinical methods

The calves were weighed using a scale on the day of birth as well as on days 21, 42 and 63. Each calf was examined daily by inspection for any clinical symptoms in the morning throughout the study period. Blood samples were collected from each calf by puncture of the Vena jugularis on day 4; serum was isolated by centrifugation and stored at −20 °C until examination. A total of 17 fecal samples were rectally collected from each calf (in the morning of days 4–14, 16, 18, 21, 28, 42 and 63), labeled and kept at + 4 °C until examination. The consistency of fecal samples was estimated by scoring (solid = score 0, semi-liquid = 1, and watery = 2; Joachim et al., 2003). Two of the four calves that died during the study were necropsied by an external laboratory.

2.6. Laboratory methods

2.6.1. Serum total protein concentration

To determine the efficacy of passive transfer of immunity serum total protein concentration (g/l) was estimated using a hand refractometer (Weaver et al., 2000).

2.6.2. Cryptosporidium

The negative carbol-fuchsin fecal smear staining method was used to detect *Cryptosporidium* oocysts (Heine, 1982; Potters and Van Esbroeck, 2010) in fecal samples of days 4–21. The intensity of oocyst shedding was estimated by scoring (scores 0–4) according to Castro-Hermida et al. (2002). For the detection of *Cryptosporidium* spp. coproantigen, a commercially available immunochromatographic in-situ rapid diagnostic kit (SmartStrips™ Cryptosporidium-Bio K403 in combination with SmartStrips™ App; both: BioX Diagnostics, Rochefort, Belgium) was used according to the manufacturer's instructions. This test allows a semiquantitative estimation of the intensity of *Cryptosporidium* infection by scoring (scores 0-4).

For the molecular characterization of the *Cryptosporidium* species and *C. parvum* genotype present on the farm, DNA was isolated from 10 oocyst-positive fecal samples of different calves using QIAamp DNA

Stool kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer´s protocol. The small-subunit ribosomal RNA (SSU rRNA) gene was partially amplified by PCR, and restriction fragment length polymorphism (RFLP) analysis was performed as described by Xiao et al. (1999). To identify the *C. parvum* subtype, DNA was isolated, and a 400 bp-fragment of the glycoprotein 60 (gp60) gene was amplified by nested PCR using primers AL3531 and AL3533 in the first PCR and AL3532 and LX0029 in the second PCR as described by Sulaiman et al. (2005). The amplicons were purified and directly sequenced using the primers of the second PCR through an external service provider (LGC Genomics GmbH, Berlin, Germany). The gp60 sequences obtained were assembled and a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the GenBank database was performed. Subtypes of *C. parvum* were designated according to the nomenclature described by Sulaiman et al. (2005), i. e., the number of the tandem trinucleotide repeats TCA (A), TCG (G), and TCT (T) coding for the amino acid serine and the number of the repeat ACATCA (R) immediately after the nucleotide triplets.

2.6.3. Rotavirus, Coronavirus and Escherichia coli K99

For the qualitative detection of Rotavirus, Coronavirus and/or *E. coli* K99 coproantigens in all fecal samples of days 4–21, a commercially available immunochromatographic rapid diagnostic kit (Rainbow Calf Scours-Bio K288; BioX Diagnostics, Rochefort, Belgium) was used according to the manufacturer's instructions.

2.6.4. Eimeria, Giardia and other parasites

On day 63, a fecal sample of each calf was qualitatively examined for *Eimeria* spp., *Giardia* sp. and any other parasite stages, e.g., *Entamoeba* cysts and *Strongyloides* eggs, using the modified formalin-ether sedimentation technique (Young et al., 1979). Additionally for *Giardia* spp. coproantigen, a commercially available qualitative enzyme immunoassay (ProspecT™ *Giardia* Microplate Assay, Virotech Diagnostics GmbH, Rüsselsheim, Germany) was used according to the manufacturer's instructions.

2.7. Statistical analyses

Statistical analyses were performed using the statistical program packages BMDP (Dixon, 1993) and BIAS (Ackermann, 2010). For the description of parameters the respective arithmetic mean, standard deviation, minimum and maximum were calculated. Differences of the detection of any pathogen between the 3 treatment groups were analyzed for significance using Chi^2-test (dichotomous or nominal scaled variables) or Kruskal-Wallis test with Bonferroni corrected Dunn's post-test (ordinal scaled parameters). To get a global basis for evaluation the intensity of the *Cryptosporidium* infection was estimated using the 'area under the curve' (AUC) of both the oocyst shedding and coproantigen scores over the time. In a first step, the AUC of the respective scores was calculated for each calf during days 4–21. In a second step, a statistical comparison was done for the individual AUC values using the one-way analysis of covariance with repeated measures with respect to the treatment group, having the grouped number of parturitions of cows as a covariate; in case of an overall significant difference, a pairwise comparison of treatments followed using the Student-Newman-Keuls-test. In addition to the AUC analysis for oocyst and coproantigen scores, a two-way analysis of covariance with repeated measures with respect to the treatment group and day of observation, again having the grouped number of parturitions of cows as covariate was done (because the scores were not metrically scaled and normally distributed, the results must be seen as exploratory ones). The aim of this additional comparison was to examine the time effect. Correlation analyses were done to analyze the relationship between the number of days with diarrhea and serum total protein concentrations, as well as AUC values of *Cryptosporidium* oocyst shedding or coproantigen scores. Kappa statistic was calculated to assess the agreement between the results of the two methods used for the detection of *Cryptosporidium* infection. Additionally, the correlation of scoring results of both these methods was evaluated using Spearman's rank correlation coefficient (r_s) with respect to the scale of the parameters. All the statistical tests were two-tailed, and differences or effects with p-values ≤ 0.05 were considered as significant.

3. Results

3.1. Lactation number of cows

The study calves were born from primiparous, secundiparous or multiparous cows. The average lactation number of cows was 1.15 ± 1.34 and did not differ significantly among the treatment groups ($p = 0.822$).

3.2. Serum total protein concentration

The serum total protein concentration on day 4 reached or exceeded the value of 55 g/l in 118 calves; in 3 and 2 calves it was 50 g/l and 54 g/l, respectively. It was not influenced by the number of parturitions of dams and did not differ significantly among the treatment groups (CON: 63.3 ± 4.9 g/l; SCFP: 62.4 ± 6.1 g/l; HALO: 61.7 ± 5.5 g/l).

3.3. Cryptosporidium infection

3.3.1. Species, genotype and subtype

The *Cryptosporidium* species isolated from 10 different calf fecal samples was identified by PCR-RFLP analysis as *C. parvum*, genotype B, and based on the gp60 gene the isolates belonged to subtype IIaA15G2R1.

3.3.2. Comparison of methods

For all 1713 fecal samples examined the results (positive vs. negative) of the coproantigen immunochromatography substantially agreed with those of the carbol-fuchsin fecal smear staining (kappa value: 0.85; 95% confidence interval: 0.80–0.89). The relative sensitivity and relative specificity of the staining method was 88.9% (86.5–91.0%) and 95.3% (93.7–96.5%), respectively, employing the immunochromatography as relative gold standard. The scoring results of both methods were highly correlated ($r_s = 0.867$; $p < 0.001$).

3.3.3. Prevalence

Almost all study calves, regardless of the treatment group, shed *Cryptosporidium* oocysts (98.4%) and were positive for coproantigen (99.2%) at least once during their first 3 weeks of life (Table 1). In total, 726 (42.4%) and 766 (44.7%) of the 1713 fecal samples examined on days 4–21 were positive for oocysts or coproantigen, respectively. The number of oocyst- and coproantigen-positive fecal samples in the treatment groups differed significantly ($p = 0.033$ and $p = 0.006$,

Table 1
Number of calves being *Cryptosporidium*-positive at least once and number of positive fecal samples during days 4–21 post-partum.

Parameter	Treatment group	N[1] calves	N[1] (%) fecal samples
Oocysts	CON	41/41[a]	259/571 (45.4)[a]
	SCFP	41/41[a]	258/572 (45.1)[a]
	HALO	40/41[a]	209/570 (36.1)[b]
Coproantigen	CON	40/41[a]	263/571 (46.1)[a]
	SCFP	41/41[a]	278/572 (48.6)[a]
	HALO	40/41[a]	225/570 (39.5)[b]

Values of a parameter with different superscripts in a column are significantly different ($p < 0.01$); in all other cases values in a column are not significantly different ($p > 0.05$).

[1] Number of positive calves or samples/total number of calves or samples.

Table 2
First and last day as well as duration of detection of *Cryptosporidium* oocyst shedding and coproantigen in calves.

Parameter	Treatment group	First detection on day[1]		Last detection on day[1]		Duration (days)	
		Mean (SD)[2]	Min-max[3]	Mean (SD)	Min-max	Mean (SD)	Min-max
Oocysts	CON	9.9 (2.5)[a]	6–18	17.7 (2.2)[a]	13–21	8.5 (2.5)[a]	4–16
	SCFP	9.7 (2.1)[a]	6–16	16.8 (2.1)[a]	12–21	8.1 (2.0)[a]	3–13
	HALO	11.1 (2.3)[a]	6–18	17.9 (2.2)[a]	13–21	7.7 (2.7)[a]	0–16
Coproantigen	CON	9.3 (2.9)[a]	4–18	17.6 (2.5)[a]	13–21	9.0 (3.3)[a]	0–16
	SCFP	9.4 (2.3)[a]	4–16	17.4 (2.4)[a]	12–21	9.0 (2.6)[a]	3–13
	HALO	11.0 (2.9)[a]	6–18	18.2 (2.4)[a]	13–21	8.0 (3.3)[a]	0–16

Values of a parameter with the same superscript in a column are not significantly different ($p > 0.05$.
[1] Day 0 and 21 were the day of birth and last day of fecal examination, respectively.
[2] arithmetic mean (standard deviation).
[3] minimum–maximum.

Table 3
Intensity of *Cryptosporidium* infection in calves estimated by AUC values of oocyst shedding scores and coproantigen scores during days 4–21 post-partum.

Treatment group	Mean (SD)[1] of AUC values	
	Oocyst scores	Coproantigen scores
CON	17.0 (6.2)[a]	17.1 (6.5)[a]
SCFP	14.7 (5.6)[a,b]	14.6 (4.2)[b]
HALO	13.1 (6.3)[b]	13.1 (5.0)[b]

Values with different superscripts in a column are significantly different ($p < 0.05$ and $p < 0.01$ for oocysts and coproantigen, respectively).
[1] Arithmetic mean (standard deviation).

respectively): it was significantly lower in HALO treated calves than in those of the CON ($p = 0.003$ and $p = 0.029$, respectively) and SCFP fed calves ($p = 0.005$ and $p = 0.002$, respectively). There was no significant difference between CON and SCFP (Table 1).

3.3.4. Onset and duration

Cryptosporidium oocysts and coproantigen were first detected in feces on day 6 and 4, respectively. There was a trend of a later onset of oocyst shedding and coproantigen positivity in HALO treated calves than in those of the other treatment groups (Table 2). The individual duration of oocyst shedding or coproantigen positivity ranged from 0 to 16 days and 0 to 18 days, respectively, without significant differences among the treatment groups (Table 2).

3.3.5. Intensity

The AUC values of both the oocyst shedding and coproantigen scores over time were used to estimate the intensity of the *Cryptosporidium* infection. The overall comparison of both AUC values showed a significant difference among the 3 treatment groups (oocyst shedding: $p = 0.013$; coproantigen: $p = 0.0062$). In the subsequent pairwise comparison, the AUC values of oocyst shedding scores were significantly lower in HALO treated calves (0.77 times, $p < 0.01$) than in CON ones, and the AUC values of coproantigen scores were significantly lower in both HALO treated (0.77 times, $p < 0.01$) and SCFP fed calves (0.85 times, $p < 0.05$) than in CON ones (Table 3).

The two-way analysis of covariance did not show any significant effect of the age of cows (first parturition vs. second parturition, or first parturition vs. > 2 parturitions) on the *Cryptosporidium* oocyst shedding scores. However, the mean oocyst shedding score was significantly associated with the treatment ($p < 0.001$), and time ($p < 0.001$), and there was a significant treatment by time interaction ($p < 0.001$). This implies that the overall average score was different among the treatment groups, and the scores varied as calves aged. The score curves of CON and SCFP were similar and started earlier and ran higher than that of HALO (Fig. 1), however, overall means of AUC between SCFP and HALO were similar (Table 3). Very similar results were obtained for coproantigen scores (data not shown).

There was no statistically significant correlation between the AUC values of *Cryptosporidium* oocyst shedding scores ($r = 0.074$, $p = 0.427$) or coproantigen scores ($r = 0.148$, $p = 0.111$) of each calf and the serum total protein concentration measured on day 4.

3.4. Rotavirus, Coronavirus and E. coli

Rotavirus, Coronavirus or *E.coli* coproantigen was found at least once in 30.3%, 2.4% and 21.3%, respectively, of calves and in 3.5%, 0.2% and 2.3%, respectively, of the fecal samples examined on days 4–21. There was no significant difference among the treatment groups (Table 4).

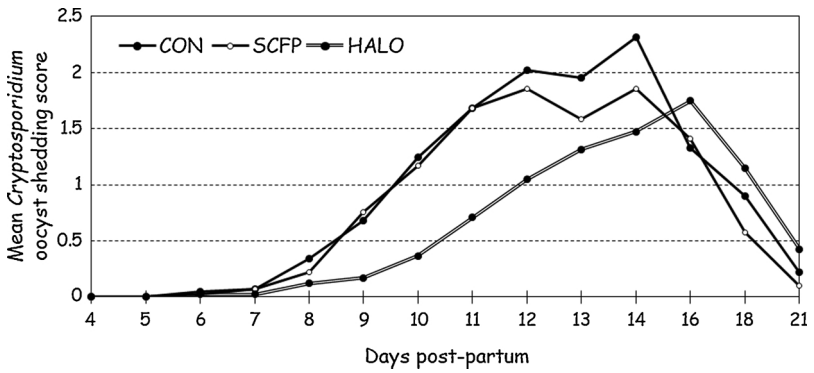

Fig. 1. Course of mean *Cryptosporidium* oocyst shedding scores (0–4) of calves daily treated with halofuginone on days 1–7 (HALO), daily fed with *S. cerevisiae* fermentation products on days 1–63 (SCFP) or remaining untreated (CON).

Table 4
Number of calves being Rotavirus, Coronavirus or *E. coli* K99 coproantigen-positive at least once and number of positive fecal samples during days 4–21 post-partum.

Coproantigen	Treatment group	N[1] (%) calves	N[1] (%) fecal samples
Rotavirus	CON	11/41 (26.8)[a]	18/571 (3.2)[a]
	SCFP	15/41 (36.6)[a]	23/572 (4.0)[a]
	HALO	11/40 (26.8)[a]	19/570 (3.3)[a]
Coronavirus	CON	0/41 (0)[a]	0/571 (0)[a]
	SCFP	1/41 (2.4)[a]	1/572 (0.2)[a]
	HALO	2/40 (4.9)[a]	2/570 (0.4)[a]
E. coli K99	CON	10/41 (24.4)[a]	13/571 (2.3)[a]
	SCFP	7/41 (17.1)[a]	12/572 (2.1)[a]
	HALO	9/40 (22.9)[a]	14/570 (2.5)[a]

Values of respective coproantigen with the same superscript in a column are not significantly different ($p > 0.05$).
[1] Number of positive calves or samples/total number of calves or samples.

Table 5
Number of calves showing diarrhea at least once, number of semi-liquid or watery fecal samples, duration of diarrhea and its intensity estimated by AUC values of the number of days with diarrhea during days 4–21 post-partum.

Treatment group	N[1] calves	N[1] (%) fecal samples	Duration (days)		Mean (SD)[2] of AUC values
			Mean (SD)	Min-max[3]	
CON	40/41[a]	167/571 (29.2)[a]	5.5 (2.6)[a]	0–11	7.9 (6.2)[a]
SCFP	40/41[a]	176/572 (30.8)[a]	5.7 (2.5)[a]	0–11	9.5 (9.3)[a]
HALO	41/40[a]	169/570 (29.6)[a]	5.7 (2.5)[a]	1–11	8.3 (6.4)[a]

Values with the same superscript in a column are not significantly different ($p > 0.05$).
[1] Number of positive calves or samples/total number of calves or samples.
[2] arithmetic mean (standard deviation).
[3] minimum–maximum.

3.5. Mortality

Four calves having diarrhea died during the study period (3.3% mortality): one calf from each treatment groups CON and SCFP (on day 15 and 18 day, respectively), and two HALO treated calves (on days 14 and 42). The SCFP calf and one of the HALO calves were post-mortem examined by an external laboratory; the pathological diagnosis was 'bacteria- and/or virus-caused enteritis' and '*Candida*-caused enteritis', respectively.

3.6. Diarrhea

3.6.1. Frequency, duration and intensity

Diarrhea (diagnosed if a sample had not solid consistency) occurred in 121 (98.4%) of the 123 calves at least once during days 4–21. In total, 29.9% of all fecal samples showed a semi-liquid or watery consistency, and the treatment groups did not differ significantly. Diarrhea lasted for 5.5–5.7 days on average (Table 5). The majority of fecal samples with altered consistency was seen between days 8 and 18 (Fig. 2). The number of days with diarrhea was not significantly correlated with its serum total protein concentration on day 4 ($r = 0.053$, $p = 0.566$). The AUC values of the number of days with diarrhea did not differ significantly among the treatment groups (Table 5).

3.6.2. Diarrhea and Cryptosporidium infection

The intensity of *Cryptosporidium* infection measured by the individual mean AUC value of coproantigen scores was significantly correlated with the number of days with diarrhea during days 4–21 ($r = 0.243$, $p = 0.007$): the higher the AUC values the more days with diarrhea were observed in mean. For oocyst shedding this correlation was not statistically significant ($r = 0.046$, $p = 0.621$).

3.7. Bodyweight

The individual birth weight of the calves ranged from 26.0 to 55.4 kg, but the mean values did not differ significantly ($p = 0.306$) among the treatment groups (Table 6). There was a highly significant group difference of the daily weight gain during the first 3 weeks of life ($p = 0.009$): HALO treated calves had a significantly lower average daily gain (ADG) than SCFP fed ($p = 0.029$) and CON calves ($p = 0.031$). There was no significant difference between SCFP and CON calves. However, no significant difference in ADG was observed among the treatment groups for the rest of the study period. At the end of the 9-week study period the individual total weight gain ranged from 15.5 to 41.1 kg, and the ADG was 581 ± 84 g; it was slightly but not significantly lower in HALO treated calves than in calves of the other treatment groups (Table 6).

3.8. Eimeria, Giardia and other parasites

On day 63, 15 (12.9%) of the 116 calves examined were positive for *Eimeria* (mainly *E. bovis*) oocysts, 32 (27.6%) for *Giardia* (cysts or coproantigen) and 35 (30.2%) for *Entamoeba* cysts without any significant differences among the treatment groups. No stages of other parasites were found.

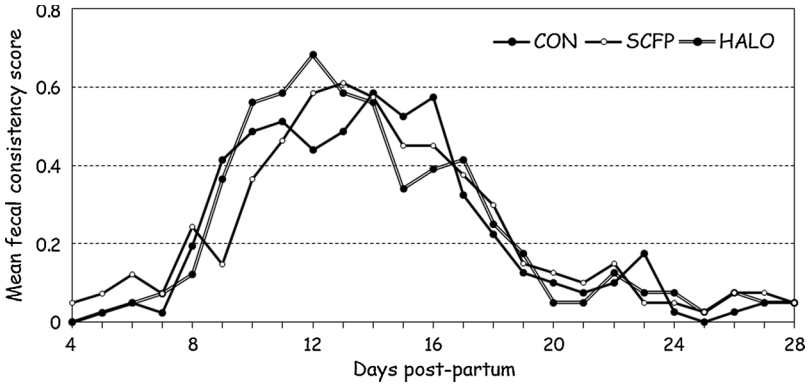

Fig. 2. Course of mean fecal consistency scores (score 0: solid; 1: semi-liquid; 2: watery) of calves treated with halofuginone on days 1–7 (HALO), daily fed with *S. cerevisiae* fermentation products on days 1–63 (SCFP) or remaining untreated (CON).

Table 6
Birth weight and bodyweight gain of calves during the first 63 days of life.

Treatment group	Mean (SD)[1] birth weight (kg)	Mean (SD) daily weight gain (g)				Mean (SD) final weight (kg)
	Day 0	Days 0–21	Days 21–42	Days 42–63	Days 0–63	Day 63
CON (N = 40)[2]	39.9 (5.3)[a]	235 (167)[a]	733 (177)[a]	799 (248)[a]	589 (89)[a]	77.1 (7.2)[a]
SCFP (N = 40)	40.4 (4.8)[a]	228 (184)[a]	767 (217)[a]	765 (217)[a]	587 (82)[a]	77.7 (6.5)[a]
HALO (N = 39)	41.1 (6.0)[a]	127 (151)[b]	746 (215)[a]	825 (250)[a]	566 (81)[a]	77.0 (6.4)[a]

Values with different superscripts in a column are significantly different ($p < 0.01$); in all other cases values in a column are not significantly different ($p > 0.05$).
[1] Arithmetic mean (standard deviation); [2]number of calves.

4. Discussion

Currently, halofuginone is the only drug approved by the authorities in Europe for the use in newborn calves against *Cryptosporidium* infection. However, its prophylactic effect against cryptosporidiosis is equivocal (Silverlås et al., 2009). Therefore, there is a demand for alternatives. The results of an experimental study had suggested a reduction of histopathological alterations in the small intestines caused by *Cryptosporidium* in calves when SCFP had been fed (Vázquez Flores et al., 2016).The present longitudinal study was performed to evaluate, for the first time under field conditions, the effects of a long-term feeding with two non-GMO SCFP against *Cryptosporidium* infection in neonatal calves in comparison with both a prophylactically HALO treated and an untreated group. As main results, the HALO treatment showed a partial anticryptosporidial activity but not a significant clinical effect. The same findings were recorded for SCFP feeding, which was, in other words, neither better nor worse than the HALO treatment.

The calves enrolled in the study had a very similar history: all were females, from the same herd, of the same breed, kept under same management conditions and received the same basic feed. The age of their dams did not differ significantly among the treatment groups. Therefore, there was no impact of the age of dams on the serum total protein concentration, the intensity of *Cryptosporidium* infection (estimated by AUC values of oocyst shedding or coproantigen scores) nor the intensity of diarrhea (estimated by AUC values of the number of days with diarrhea) of the respective calves. In almost all calves the serum total protein concentration reached or exceeded the value of 55 g/l on day 4, regardless of the treatment group. This indicates a sufficient transfer of immunoglobulins by colostrum to calves (Weaver et al., 2000), which is principally necessary to protect calves from morbidity (Urie et al., 2018c). It was, therefore, not surprising that the serum total protein concentration was not correlated with the intensity of *Cryptosporidium* infection (measured by AUC values of oocyst shedding or coproantigen scores) or the number of days with diarrhea in this study.

4.1. Cryptosporidium infection

A *Cryptosporidium* infection was diagnosed in almost all calves in all treatment groups. In untreated control calves nearly half the fecal samples collected during the first 3 weeks of life were positive for oocysts and coproantigen. In contrast, co-infections with other enteropathogens (Rotavirus, Coronavirus and *E. coli*) were detected in a smaller number of calves, and most of them were positive on one day only, resulting in only few positive fecal samples. This is in accordance with results from previous studies (e.g., Gillhuber et al., 2014; Al Mawly et al., 2015; Anonymous, 2017). Therefore, *Cryptosporidium* was considered to be the main enteropathogen and responsible for the episodes of diarrhea in the present study. In untreated calves *Cryptosporidium* oocyst shedding started 9.9 days after birth on average, which is in accordance with previous field observations (*e.g.*, Castro-Hermida et al., 2002; Niine et al., 2018; Urie et al., 2018a). It is also similar to data from experimental infections (Zambriski et al., 2013) showing that the study calves became infected shortly after birth. The species was identified as *C. parvum* genotype B, subtype IIaA15G2R1 that is commonly found in neonatal calves in Germany (Broglia et al., 2008; Holzhausen et al., 2019) and elsewhere (Feng et al., 2018).

The sensitivity of the direct fecal smear staining was lower compared with a coproantigen detection method used as gold standard, a finding already reported previously (*e.g.*, Chartier et al., 2013). However, the results of both methods substantially agreed indicating that the fecal smear staining can be used as a simple, fast and inexpensive method for the *Cryptosporidium* diagnosis, at least in endemically infected cattle farms.

4.2. Effect of halofuginone on Cryptosporidium infection

The 7-day prophylactic HALO treatment neither prevented the *Cryptosporidium* infection nor reduced the number of *Cryptosporidium*-positive calves. However, HALO treatment resulted in a significant reduction of the number of *Cryptosporidium*-positive fecal samples as compared with both the CON and SCFP fed calves. There was also a trend, being not significant, for a later onset of oocyst and coproantigen positivity in HALO treated calves than in the two other groups. Finally, the intensity of the *Cryptosporidium* infection (estimated by AUC values of oocyst shedding or coproantigen scores) was significantly reduced in the HALO group in comparison with CON but not with SCFP. All in all, these results indicate some but not fully prophylactic anticryptosporidial effect of HALO, as already observed in previous studies (e.g., Silverlås et al., 2009; Trotz-Williams et al., 2011; Delafosse et al., 2015; Niine et al., 2018).

4.3. Effect of S. cerevisiae fermentation products on Cryptosporidium infection

Daily feeding with the two SCFP for 9 weeks reduced neither the number of *Cryptosporidium*-positive calves nor the number of positive fecal samples. It did also not delay the onset or shorten the duration of oocyst and coproantigen positivity in comparison to CON. However, it was interesting to note that the intensity of the infection estimated by AUC values of coproantigen scores (but not of oocyst shedding scores) was significantly lower in SCFP fed calves than in CON ones. This finding may indicate some, although partial effect of the SCFP on the endogenous multiplication of *Cryptosporidium*, possibly resulting from a modified intestinal environment. Assuming this is correct, a reduced endogenous multiplication of the parasite would result in less pathological alterations in the intestinal mucosa corresponding with the histological findings by Vázquez Flores et al. (2016), albeit obviously without clinical improvement. In this context it is of interest to mention that SCFP had been shown to have a positive effect on the intestinal development of preweaned calves (Xiao et al., 2016) and a positive immunomodulatory effect in animals (Park, 2014; Chou et al., 2017). Of course, it warrants to further investigate the mechanism of action of SCFP.

4.4. Bodyweight gain and diarrhea

In the present study relatively low daily weight gains, ranging from

566 to 589 g in group average, were recorded at the end of the 9-week period. This may be a negative consequence of the diarrheal episodes during the first several weeks of life in more or less all calves. The number of diarrheal days was significantly correlated with the intensity of the *Cryptosporidium* infection indicating that the parasite was responsible for diarrhea in the present study. In contrast, a considerably higher ADG (700 g) at an average weaning age of 66 days was reported for dairy calves in a large-scale US study; however, here clinical signs had been seen in only 38% of the animals, including 21% with digestive signs (Urie et al., 2018b).

Although the HALO treatment showed a partial anticryptosporidial activity, it had no clinical effect in the present study: it did not significantly affect the onset, nor the frequency, duration and intensity of diarrhea, the main clinical symptom of cryptosporidiosis. These results are consistent with observations in previous studies reporting a non-significant association between its prophylactic use and the incidence, duration or intensity of diarrhea (Silverlås et al., 2009; Trotz-Williams et al., 2011; Almawly et al., 2013; Meganck et al., 2015). The same findings were recorded for SCFP feeding, which was, in other words, neither better nor worse than the HALO treatment.

A non-significant difference of weight gains between HALO treated and CON calves had been reported by others (Trotz-Williams et al., 2011), and in a more recent study the HALO treatment even had a negative effect on the daily weight gain after 3 months (Niine et al., 2018). It should be stressed that in the present study the 7-day HALO treatment clearly resulted in a negative impact on the weight gain during the first 3 weeks of life as compared SCFP or CON calves. This temporarily insufficient weight development can be explained by the known toxicity of HALO that can induce reversible gastro-intestinal inflammatory or necrotic lesions also at therapeutic doses (Anonymous, 2000).

4.5. *Eimeria, Giardia and other parasites*

Some calves from each treatment group were positive for *Eimeria* spp. oocysts, *Giardia* and/or *Entamoeba* cysts at 9 weeks of age. Patent *Eimeria* infections are occasionally seen at an age of 3 weeks but still considered clinically inapparent (e.g., Faber et al., 2002). Severe *Eimeria* infection are first observed in older, group-housed calves (Niine et al., 2018) and may then result in morbidity (Bangoura et al., 2012). *Giardia* spp. infections are very common in cattle farms worldwide. Calves usually become infected before the 5th week of life, and prevalence peaks were reported in 1- to 6-month old animals (Geurden et al., 2010 2012; Gillhuber et al., 2014; Niine et al., 2018; Urie et al., 2018a). *Giardia* spp. seem to be able to induce diarrhea (Gillhuber et al., 2014) and may reduce weight gain (Urie et al., 2018a) mainly in calves older than one month. *Entamoeba bovis* infections are not uncommon in cattle but are considered less pathogenic (Matsubayashi et al., 2018).

5. Conclusions

A pre-weaning supplementation with the two *S. cerevisiae* fermentation feed additives showed very similar clinical results and weight gains in *Cryptosporidium* infected newborn calves as a 7-day halofuginone treatment under the conditions on this farm. This suggests that feeding with these *S. cerevisiae* fermentation products may be from the clinical point of view a natural alternative measure, instead of halofuginone treatment, in bovine cryptosporidiosis.

Conflict of interest statement

This study was funded through a grant by Diamond V, Cedar Rapids, Iowa, USA. PZ and IY are current employees of Diamond V.

Acknowledgements

We gratefully acknowledge the owner of the dairy farm for allowing us to access to the facilities and to perform this study. We also thank the farm personnel for their helpful collaboration as well as Dr. Jörg Hirzmann for his help and advice on the molecular characterization of the *Cryptosporidium* species.

References

Ackermann, H., 2010. Bias für Windows, version 9.05. Epsilon Hochheim.
Al Mawly, J., Grinberg, A., Prattley, D., Moffat, J., French, N., 2015. Prevalence of endemic enteropathogens of calves in New Zealand dairy farms. N.Z. Vet. J. 63, 147–152.
Almawly, J., Prattley, D., French, N.P., Lopez-Villalobos, N., Hedgespeth, B., Grinberg, A., 2013. Utility of halofuginone lactate for the prevention of natural cryptosporidiosis of calves, in the presence of co-infection with rotavirus and Salmonella typhimurium. Vet. Parasitol. 197, 59–67.
Anonymous, 2000. European Agency for the Evaluation of Medical Products. Halofuginone – Summary Report 2. EMEA/MRL/741/00-FINAL June 2000. . http://www.ema.europa.eu/ema/).
Anonymous, 2017. Northern Ireland disease surveillance report, January to March 2017. Vet. Rec. 180, 490–494.
Bangoura, B., Mundt, H.-C., Schmäschke, R., Westphal, B., Daugschies, A., 2012. Prevalence of *Eimeria bovis* and *Eimeria zuernii* in German cattle herds and factors influencing oocyst excretion. Parasitol. Res. 110, 875–881.
Broglia, A., Reckinger, S., Cacció, S.M., Nöckler, K., 2008. Distribution of *Cryptosporidium parvum* subtypes in calves in Germany. Vet. Parasitol. 154, 8–13.
Castro-Hermida, J.A., González-Losada, Y.A., Mezo-Menéndez, M., Ares-Mazás, E., 2002. A study of cryptosporidiosis in a cohort of neonatal calves. Vet. Parasitol. 106, 11–17.
Cavalier-Smith, T., 2014. Gregarine site-heterogeneous 18S rDNA trees, revision of gregarine higher classification, and the evolutionary diversification of Sporozoa. Eur. J. Protistol. 50, 472–495.
Chartier, C., Rieux, A., Delafosse, A., Lehebel, A., Paraud, C., 2013. Detection of *Cryptosporidium* oocysts in fresh calf faeces: characteristics of two simple tests and evaluation of a semi-quantitative approach. Vet. J. 198, 148–152.
Chou, W.K., Park, J., Carey, J.B., McIntyre, D.R., Berghman, L.R., 2017. Immunomodulatory effects of *Saccharomyces cerevisiae* fermentation product supplementation on immune gene expression and lymphocyte distribution in immune organs in broilers. Front. Vet. Sci. 4, 37.
De Waele, V., Speybroeck, N., Berkvens, D., Mulcahy, G., Murphy, T.M., 2010. Control of cryptosporidiosis in neonatal calves: use of halofuginone lactate in two different calf rearing systems. Prev. Vet. Med. 96, 143–151.
Delafosse, A., Chartier, C., Dupuy, M.C., Dumoulin, M., Pors, I., Paraud, C., 2015. *Cryptosporidium parvum* infection and associated risk factors in dairy calves in western France. Prev. Vet. Med. 118, 406–412.
Deplazes, P., Eckert, J., Mathis, A., Von Samson-Himmelstjrna, G., Zahner, H., 2016. Parasitology in Veterinary Medicine. Wageningen Academic Publ. 122–127.
Dixon, W.J., 1993. BMDP Statistical Software, Berkeley.
Faber, J.-E., Kollmann, D., Heise, A., Bauer, C., Failing, K., Bürger, H.-J., Zahner, H., 2002. *Eimeria* infections in cows in the periparturient phase and their calves: oocyst excretion and levels of specific serum and colostrum antibodies. Vet. Parasitol. 104, 1–17.
Fayer, R., 2008. General biology. In: Fayer, R., Xiao, L. (Eds.), *Cryptosporidium* and Cryptosporidiosis, 2nd ed. CRC Press, Boca Baton, pp. 1–42.
Fayer, R., Ellis, W., 1993. Paromomycin is effective as prophylaxis for cryptosporidiosis in dairy calves. J. Parasitol. 79, 771–774.
Feng, Y., Ryan, U.M., Xiao, L., 2018. Genetic diversity and population structure of Cryptosporidium. Trends Parasitol. 34, 997–1011.
Vázquez Flores, S., Jésus Guerrero Carrillo, M., Carrillo, M., Scott, M.F., Hamann, J., Barrera Almanza, S., Guizar Bravo, C., Baños Quintana, A.P., Aranda Vargasb, P.J., 2016. Effects of *Saccaromyces cerevisiae* fermentation products on intestinal villi integrity in neonatal calves naturally infected with *Cryptosporidium* spp. J. Anim. Sci. 94 (E-Suppl. 5), 714–715.
Geurden, T., Vercruysse, J., Claerebout, E., 2010. Is *Giardia* a significant pathogen in production animals? Exp. Parasitol. 124, 98–106.
Geurden, T., Vanderstichel, R., Pohle, H., Ehsan, A., von Samson-Himmelstjerna, G., Morgan, E.R., Camuset, P., Capelli, G., Vercruysse, J., Claerebout, E., 2012. A multicentre prevalence study in Europe on *Giardia duodenalis* in calves, with molecular identification and risk factor analysis. Vet. Parasitol. 190 (383), 390.
Gillhuber, J., Rügamer, D., Pfister, K., Scheuerle, M.C., 2014. Giardiosis and other enteropathogenic infections: a study on diarrhoeic calves in Southern Germany. BMC Res. Notes 7, 112.
Grinberg, A., Markovics, A., Galindez, J., Lopez-Villalobos, N., Kosak, A., Tranquillo, V.M., 2002. Controlling the onset of natural cryptosporidiosis in calves with paromomycin sulphate. Vet. Rec. 151, 606–608.
Heine, J., 1982. Eine einfache Nachweismethode für Kryptosporidien im Kot. Zbl. Veterinärmed. B 29, 324–327.
Holzhausen, I., Lendner, M., Göhring, F., Steinhöfel, I., Daugschies, A., 2019. Distribution of *Cryptosporidium parvum* gp60 subtypes in calf herds of Saxony. Germany. Parasitol. Res. in press. https://doi.org/10.1007/s00436-019-06266-1.
Joachim, A., Krull, T., Schwarzkopf, J., Daugschies, A., 2003. Prevalence and control of bovine cryptosporidiosis in German dairy herds. Vet. Parasitol. 112, 277–288.

Matsubayashi, M., Matsuura, Y., Nukata, S., Daizi, Y., Shibahara, T., Teramoto, I., Matsuo, T., Uni, S., Hatta, T., Kaneko, A., Tsuji, N., Sasai, K., 2018. First detection and molecular identification of *Entamoeba bovis* from Japanese cattle. Parasitol. Res. 117, 339–342.

Meganck, V., Hoflack, G., Piepers, S., Opsomer, G., 2015. Evaluation of a protocol to reduce the incidence of neonatal calf diarrhoea on dairy herds. Prev. Vet. Med. 118, 64–70.

Niine, T., Dorbek-Kolin, E., Lassen, B., Orro, T., 2018. *Cryptosporidium* outbreak in calves on a large dairy farm: effect of treatment and the association with the inflammatory response and short-term weight. Res. Vet. Sci. 117, 200–208.

Park, J.-W., 2014. Effects of Yeast Product on Modulating the Adaptive Immune Function in Broilers. M. Sc. Thesis. Texas A & M University, College Station, pp. 1–45. http://hdl.handle.net/1969.1/153890).

Potters, I., Van Esbroeck, M., 2010. Negative staining technique of Heine for the detection of *Cryptosporidium* spp.: a fast and simple screening technique. Open Parasitol. J. 4, 1–4.

Shahiduzzaman, M., Daugschies, A., 2012. Therapy and prevention of cryptosporidiosis in animals. Vet. Parasitol. 188, 203–214.

Silverlås, C., Björkman, C., Egenvall, A., 2009. Systematic review and meta-analyses of the effect of halofuginone against calf cryptosporidiosis. Prev. Vet. Med. 91, 73–84.

Stockdale, H.D., Spencer, J.A., Blagburn, B.L., 2008. Prophylaxis and chemotherapy. In: Fayer, R., Xiao, L. (Eds.), *Cryptosporidium* and Cryptosporidiosis, 2nd ed. CRC Press, Boca Raton, pp. 255–287.

Sulaiman, I.M., Hira, P.R., Zhou, L., Al-Ali, F.M., Al-Shelahi, F.A., Shweiki, H.M., Iqbal, J., Khalid, N., Xiao, L., 2005. Unique endemicity of cryptosporidiosis in children in Kuwait. J. Clin. Microbiol. 43, 2805–2809.

Thompson, R.C.A., Koha, W.H., Clode, P.L., 2016. *Cryptosporidium* – what is it? Food Waterborne Parasitol. 4, 54–61.

Thomson, S., Hamilton, C.A., Hope, J.C., Katzer, F., Mabbott, N.A., Morrison, L.J., Innes, E.A., 2017. Bovine cryptosporidiosis: impact, host-parasite interaction and control strategies. Vet. Res. 48, 42.

Trotz-Williams, L.A., Martin, S.W., Leslie, K.E., Duffield, T., Nydam, D.V., Peregrine, A.S., 2007. Calf-level risk factors for neonatal diarrhea and shedding of *Cryptosporidium parvum* in Ontario dairy calves. Prev. Vet. Med. 82, 12–28.

Trotz-Williams, L.A., Jarvie, B.D., Peregrine, A.S., Duffield, T.F., Leslie, K.E., 2011. Efficacy of halofuginone lactate in the prevention of cryptosporidiosis in dairy calves. Vet. Rec. 168, 509.

Urie, N.J., Lombard, J.E., Shivley, C.B., Adams, A.E., Kopral, C.A., Santin, M., 2018a. Preweaned heifer management on US dairy operations: part III. Factors associated with *Cryptosporidium* and *Giardia* in preweaned dairy heifer calves. J. Dairy Sci. 101, 9199–9213.

Urie, N.J., Lombard, J.E., Shivley, C.B., Kopral, C.A., Adams, A.E., Earlywine, T.J., Olson, J.D., Garry, F.B., 2018b. Preweaned heifer management on US dairy operations: part I. Descriptive characteristics of preweaned dairy heifer raising practices. J. Dairy Sci. 101, 9168–9184.

Urie, N.J., Lombard, J.E., Shivley, C.B., Kopral, C.A., Adams, A.E., Earlywine, T.J., Olson, J.D., Garry, F.B., 2018c. Preweaned heifer management on US dairy operations: part V. Factors associated with morbidity and mortality in preweaned dairy heifer calves. J. Dairy Sci. 101, 9229–9244.

Weaver, D.M., Tyler, J.W., VanMetre, D.C., Hostetler, D.E., Barrington, G.M., 2000. Passive transfer of colostral immunoglobulins in calves. J. Vet. Intern. Med. 14, 569–577.

Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R.C.A., Fayer, R., Lal, A.A., 1999. Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. Appl. Environ. Microbiol. 65, 3386–3391.

Xiao, J.X., Alugongo, G.M., Chung, R., Dong, S.Z., Li, S.L., Yoon, I., Wu, Z.H., Cao, Z.J., 2016. Effects of *Saccharomyces cerevisiae* fermentation products on dairy calves: ruminal fermentation, gastrointestinal morphology, and microbial community. J. Dairy Sci. 99, 5401–5412.

Young, K.H., Bullock, S.L., Melvin, D.M., Spruill, C.I., 1979. Ethyl acetate as a substitute for diethyl ether in the formalin-ether sedimentation technique. J. Clin. Microbiol. 10, 852–853.

Zambriski, J.A., Nydam, D.V., Bowman, D.D., Bellosa, M.L., Burton, A.J., Linden, T.C., Liotta, J.L., Ollivett, T.L., Tondello-Martins, L., Mohammed, H.O., 2013. Description of fecal shedding of *Cryptosporidium parvum* oocysts in experimentally challenged dairy calves. Parasitol. Res. 112, 1247–1254.