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Effects of recycled manure solids bedding on the spread of gastrointestinal parasites in the environment of dairies and milk

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ABSTRACT

The primary aim of this work was to isolate common bovine digestive tract parasites in recycled manure bedding (RMS), as well as to determine the ability of current RMS preparation procedures to eliminate these pathogens. Other objectives were to assess whether any of the aforementioned parasites could be retrieved in bulk milk from dairies using RMS and to study whether the prevalence of these parasites differed among manure of cows housed on RMS versus on straw bedding. For the study, 27 RMS farms and 61 control farms were recruited. Samples of manure from the pre-pit and milk from the bulk tank were recovered from straw-bedding farms and RMS-based farms. In addition, samples from the manure solid fraction after liquid extraction, RMS before use, and RMS currently in use were recovered from RMS herds. Parasites were first detected by double centrifugation zinc sulfate flotation to enhance isolation of gastrointestinal protozoa, and by modified Wisconsin sugar flotation for the appraisal of gastrointestinal nematodes. *Cryptosporidium* parasites were confirmed by nested PCR amplification and sequencing of a portion of the gene encoding the small subunit rRNA. Results revealed a high prevalence of *Cryptosporidium* spp. (*C. parvum*, *C. andersoni*, and *C. meleagridis*, identified by PCR) and *Eimeria* spp. (mainly *E. bovis* and *E. zuernii*) parasites in both types of farms, with a larger proportion of manure samples from RMS-bedded farms testing positive for *Cryptosporidium* parasites compared with manure from straw-bedded farms. Both *Cryptosporidium* spp. and *Eimeria* spp. oocysts were found at every step of RMS preparation and transformation, showing that current RMS preparation strategies do not guarantee the destruction of protozoan parasites. *Cryptosporidium parvum*, a potential zoonotic risk for professionals in close contact with livestock, was found to be present in 32 out of 61 straw-bedded and 24 of 27 RMS farms. No protozoan parasites were found in any sample derived from bulk milk, neither by microscopy analysis nor by molecular methods.

Key words: dairy cattle, recycled manure solids, protozoan parasites

INTRODUCTION

Recycled manure solids (**RMS**) have been used as a bedding material since the 1970s in North America (Carroll and Jasper, 1978). In the last few years, RMS has attracted the interest of dairy producers in Canada as an alternative to straw bedding, due to its on-site availability and its association with increased cow comfort (Husfeldt et al., 2012; Leach et al., 2015). Different approaches have been developed to produce RMS (Fournel et al., 2019); however, significant unknowns remain with respect to associated biological risks. Research on the effects of RMS on animal health and welfare is still sparse, and the only disease for which the consequences have been studied in detail is bacteria-borne mastitis. Studies have shed light on the bacterial populations in RMS, present even after composting, including *Klebsiella* spp., *Pseudomonas* spp., and *Escherichia coli* (Cole and Hogan, 2016; Rowbotham and Ruegg, 2016). Moreover, a recent study demonstrated that bacterial counts of mastitis pathogens in composted RMS were comparable with those in fresh recycled manure when used as freestall bedding (Cole and Hogan, 2016).

Most dairy operations underwent a major transformation during the second half of the twentieth century,

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ending with animals housed indoors year-round, using different, continuously evolving systems. Although it is true that this change reduced the exposure of dairy cows to pasture-contaminating gastrointestinal nematode (**GIN**) parasites, protozoan parasites (mainly *Cryptosporidium* spp., *Giardia* spp., and *Eimeria* spp.) may be of increased risk to housed cattle (Vande Velde et al., 2018).

Economic losses associated with protozoan parasites have not been studied in dairy cattle, but include the cost of treatment and control of enteritis, low feed conversion, and decreased milk productivity, as well as losses due to animal deaths (Olson et al., 1995; Mark-Carew et al., 2010; Sudhakara Reddy et al., 2015; Zechner et al., 2016; Thomson et al., 2017). Importantly, current evidence indicates that ruminants are potential reservoirs of zoonotic *Cryptosporidium* (Robertson et al., 2010; Gormley et al., 2011; Pumipuntu and Piratae, 2018). Bedding made from RMS is prepared by separation of the liquid fraction of manure, sometimes followed by composting of the solid fraction. Thus, RMS could be a potential transmission source of *Cryptosporidium* (especially non-composted RMS), as infected animals can pass up to 10 billion oocysts per gram of feces (O'Handley et al., 1999). Oocysts are highly resistant in the environment due to their disulfide bond-rich wall (Carey et al., 2004).

The objective of this study was to investigate whether common bovine digestive tract parasites would survive RMS preparation procedures in Quebec's dairies and to assess whether some of these parasites, especially those with zoonotic potential such as *Cryptosporidium* spp., could be retrieved in bulk milk from dairies using RMS. A secondary objective was to appraise whether the prevalence of these parasites differed among manure of cows housed on RMS compared with that of cows housed on straw bedding.

MATERIALS AND METHODS

Study Design: Farm Recruitment and Sampling

All procedures were approved by the Animal Care and Use Committee for the Veterinary College of the Université de Montréal (protocol 17-Rech-1886). Study design was an observational cross-sectional study where both exposure (use of RMS) and outcome (parasitic burden) were evaluated on the same visit. To be included, RMS farms needed to have used RMS as the primary bedding for lactating cows for at least 6 mo before the beginning of the study. Farms also had to be located within 250 km of the research facility (Saint-Hyacinthe, QC, Canada). Potential participating RMS herds were identified by contacting equipment dealers and veterinarians, as well as via social networks, and all owners identified were contacted to verify their participation in the study. Due to regional management practices, most RMS herds also participated in regular DHI programs. However, given the relatively small number of RMS herds in the study area, RMS herds not participating in DHI program were not excluded. For comparison, farms using more conventional bedding were recruited in the same area, with the help of the Eastern Canada DHI association (Valacta Inc., Ste-Anne-de-Bellevue, QC, Canada), and were selected based on their exclusive use of straw bedding for at least 6 mo. Farm visits were conducted between January and July 2018 in Quebec and eastern Ontario. Samples of manure collected from the last indoor point before the manure pit and of milk from the bulk tank were recovered from straw-bedded farms and RMS herds. In addition, 3 extra samples were recovered from RMS herds: manure solid fraction after liquid extraction, RMS before use, and RMS currently in use. The manure solid fraction was collected immediately upon its exit from the equipment used for its extraction (screw press or roller press). Unused RMS samples were obtained directly from the equipment used to distribute the bedding (e.g., collected from the mixer wagon as it was filling the stalls). For used RMS, a random sample of 5 stalls was conducted. Two used RMS samples were collected from the back third of each stall, avoiding collecting manure piles. All used RMS samples were mixed together in a plastic bag. All samples were collected by the research team, stored on ice, and brought back to Université de Montréal research facilities, where they were separated within 24 h of collection in various subsamples for the different analyses. Briefly, 10 g of manure, 10 mL of milk, 15 g of manure solid fraction after liquid extraction, 15 g of unused RMS, and 15 g of used RMS were preserved at 4°C for parasitological analyses. Parasitological analyses were conducted in triplicate at the Diagnostic Service of the Université de Montréal (Saint-Hyacinthe, QC, Canada).

Parasitology Tests

Parasite Isolation and Microscopy Analyses. To isolate and quantitate protozoa in samples, double centrifugation zinc sulfate flotation was employed (Bukhari and Smith, 1995). Modified Wisconsin sugar flotation was used to identify GIN eggs (Dryden et al., 2005). Briefly, for protozoal isolation, 2 mL of washed samples were added to 10 mL of $ZnSO_4$ (Ricca Chemical, Arlington, TX; specific gravity 1.180) and centrifuged at 1,050 × *g* for 2 min. Following centrifugation, 2 mL of fluid from the meniscus (containing the oocysts) was removed and washed 3 times in 1× PBS (pH 7.4, with

centrifugation at 1,650 × *g* for 5 min after each wash), and the pellet was resuspended to a final volume of 1 mL of 1× PBS. For GIN isolation, samples were centrifuged at 1,650 × *g* for 5 min, resuspended in saturated sucrose (Fisher Chemical, Canada), centrifuged at 650 × g for 2 min, and flotation was performed for 1 h at room temperature. The coverslip was then removed and rinsed with 1 mL of 1 × PBS to collect the eggs. Finally, for analysis of milk samples, specimens underwent fat extraction and quantitative formalin ethyl acetate concentration (Fisher Scientific Canada, Edmonton, Alberta; Zarlenga and Trout, 2004) followed by double centrifugation and zinc sulfate flotation.

Oocysts and eggs were identified and counted using a high-throughput multimode microscope (Cytation 5, Biotek, Winooski, VT) using 20×, 40×, and 60× objectives. Additionally, when *Cryptosporidium* spp. were isolated or suspected, an additional Modified Ziehl-Neelsen microscopy was performed (Fayer et al., 2007). As parasites are not distributed homogeneously in samples, 3 independent replicates were processed and analyzed. Results of these 3 analyses were summed to report several oocysts per gram of biological materials.

DNA Extraction. To extract DNA, a modified protocol was performed using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany; Thivierge et al., 2016). Total parasites recovered from zinc sulfate flotation were resuspended in QIAmp AL buffer and submitted to 10 freeze-thaw cycles (1 min in liquid nitrogen and 2 min at 56°C per cycle). Samples were centrifuged at 16,000 × *g* for 1 min and incubated with 200 μg/mL proteinase potassium at 56°C overnight. Samples were centrifuged at 16,000 × *g* for 2 min, and 400 μL of supernatant was recovered and mixed with an equal volume of absolute ethanol before starting the spin column protocol (Qiagen). For each sample, the final elution product was recovered in a clean tube, quantified, and assessed for purity using a NanoDrop system (Thermo Fisher Scientific, Waltham, MA) and either used immediately for PCR amplification or stored at −20°C until assay.

Nested PCR and RFLP. Species of *Cryptosporidium* parasites were determined via nested PCR amplification, RFLP analyses, and sequencing of a portion of the gene encoding the small subunit rRNA (Nichols et al., 2003). The PCR products were fully sequenced in both directions, with secondary PCR primers used to produce the amplicons (forward: 5′-AAGCTCGTAGTTGGATTTCTG-3′; reverse: 5′-TAAGGTGCTGAAGGAGTAAGG-3′) at the Molecular Diagnostic Service of the Veterinary College at Université de Montréal. Each gene sequence fragment was independently compared with GenBank and CryptoDB sequences of *Cryptosporidium* spp. by Basic Local Alignment Search Tool analysis (BLAST; Benson et al., 2013). Sequences were aligned using SeqMan Pro (DNASTAR, Madison, WI). The PCR-positive specimens sequenced in the present study shared >95% identity with species AF308600, MH395839.1, and AF112574.1 in GenBank. Mixed populations within the same sample, characterized by overlaying chromatograms, were elucidated by PCR-RFLP analysis using the enzymes *Vsp*I, *Dra*I, and *Dde*I (Nichols et al., 2003).

Statistical Analyses

Parasite Survival Through RMS Preparation Procedures. First, models were generated to predict how the oocyst count for a given parasite would be affected by the type of sample analyzed (manure, manure solid fraction following separation procedures, RMS before use in the stalls, or RMS collected in stalls just before replacement). A generalized mixed model was used, with oocyst count of a given parasite as outcome, sample type as the sole fixed predictor, and a random herd effect, using a negative binomial (**NB**) distribution with a natural logarithm link. To ensure that the number of samples with a zero egg count was well modeled by the NB distribution, a Vuong test comparing the conventional NB to a zero-inflated NB distribution was conducted using the Vuong.sas macro (Vuong, 1989). Said Vuong test indicated that the NB distribution was favored compared with the zero-inflated NB; NB distributions were, therefore, used in all models. The model was as follows:

$$Egg_{ij} = Log\left(\beta_0 + \boldsymbol{\beta}_1 Sample\ \ type_{ij} + \varepsilon_{ij} + \ u_j\right),$$

where Egg_{ij}, the count of oocysts for a given parasite (total *Eimeria* spp., *Eimeria bovis*, *Eimeria zuernii*, or *Cryptosporidium* spp.), for sample i from herd j was assumed to follow an NB distribution and was linked to fixed predictors through the natural logarithmic function (Log); β_0 was the mean number of oocysts in the reference category (i.e., the intercept); $\boldsymbol{\beta}_1$ was a vector of coefficients for a set of dummy variables representing sample type (with manure as reference category); $Sample\ type_{ij}$ was the type of biologic material (manure, manure solid fraction following separation procedures, RMS before use in the stalls, or RMS collected in stalls just before replacement) for sample i from herd j; and ε_{ij} and u_j were, respectively, the observation's and herd's residuals. Using this model, the sample type β coefficients could be interpreted, after exponentiation, as the relative increase or decrease in egg count for a given biologic material compared with a reference category (manure, in this case). Alternatively, the sum of β_0 and

of a given sample type coefficient can be exponentiated to illustrate the predicted median egg count for a given biologic material. When comparing sample types with one another, a Tukey-Kramer adjustment was used, to account for multiple comparisons. Finally, in cases where the generalized mixed model could not converge, then a generalized model was used (thus ignoring clustering of observations per herd). These statistical computations were conducted using the GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) with a Gaussian quadrature estimation method using 7 quadrature points (method = quad qpoints = 7).

Next, we estimated whether egg count for a given parasite in manure would be a significant predictor of egg count in unused RMS. To achieve this, a model similar to the generalized mixed NB model previously described was used, but without the herd random effect and with the egg count in unused RMS as the outcome and the egg count in manure as the sole predictor. These statistical computations were conducted using the GENMOD procedure of SAS 9.4.

Comparative Egg Counts by Bedding Type. To investigate whether counts of oocysts in manure would differ between herds bedding cows on RMS compared with straw, only 1 sample (i.e., 1 manure sample) was available per herd. Thus, a model similar to the generalized mixed NB model previously described was used, but without the random effect of the herd, and with type of bedding (RMS vs. straw) as the sole predictor. These statistical computations were conducted using the GENMOD procedure of SAS 9.4.

RESULTS

Herd Recruitment and Sampling

We identified and contacted 49 RMS farms and 139 straw-bedded farms to obtain more information to determine their eligibility for the study and willingness to participate; 27 RMS farms and 61 straw-bedded farms were recruited for the study. Of these, 16 RMS herds and all straw-bedded herds participated in regular DHI testing. Straw-bedded farms were milking 43 to 229 cows (median 65), and RMS farms were milking 55 to 900 cows (median 111). Of the RMS farms, 24 simply extracted a fraction of the liquid (15 using a roller press system and 9 using an infinite screw system) and let the solid fraction mature in a pile or a container for various time periods (from 3 h to 3 d), with the exception of 1 farm that used the solid fraction as bedding immediately after separation. Two farms used a screw press separator to extract a fraction of the liquid and then composted the solid fraction using a mechanical drum. Finally, 1 farm used a biodigestor followed by a screw press separation process. The maturation time of the bedding was highly variable between herds (from hours to several days). Furthermore, the length of use in stalls before replacement was variable and was mainly a function of stall design (shallow vs. deep bedding). When used as shallow bedding, farmers tended to replace RMS every 12 h, and when used in deep stalls, it was added from once every other day to once every 8 d. The majority of control farms used straw as shallow bedding and therefore replaced it every 12 or 24 h.

Parasite Identification

A summary of isolated parasites is presented in Table 1. *Moniezia* spp. tapeworms (cestodes) were found in 8 out of 61 straw-bedded and 1 of 27 RMS herds. Importantly, these parasites require the presence of the intermediate host, an oribatid mite, which has been described to be found in straw bedding (Irie et al., 2013). *Toxocara* spp. were isolated from one straw-bedded farm. No other types of metazoan parasites were isolated, and bulk tank milk was negative for all species, including *Moniezia* and *Toxocara* (data not shown). Flotation analyses revealed a higher prevalence ($P < 0.01$) of *Cryptosporidium*-like structures in manure from RMS herds (23 of 27; 85.2%) than straw herds (30 of 61; 49.2%). Also, this pathogen was isolated in 22 of 27 (81.5%), 23 of 27 (85.2%), and 26 of 27 (96.3%) RMS samples collected from manure solids, bedding before use, and used bedding, respectively (Table 1). Samples containing *Cryptosporidium*-like structures were further investigated by PCR. The PCR analysis identified 3 *Cryptosporidium* species: *C. parvum* in manure of 32 out of 61 straw-bedded farms and 24 of 27 RMS farms; *C. andersoni* in 19 of 61 and 20 of 27 straw and RMS farms, respectively; and *C. meleagridis* in 4 of 61 and 1 of 27 straw and RMS farms, respectively. Isolation of both *C. parvum* and *C. andersoni* occurred in 18 of 61 and 16 of 27 straw and RMS farms, respectively. *Cryptosporidium parvum*, *C. andersoni*, and *C. meleagridis* were co-detected in 1 of 61 and 4 of 27 straw-bedded and RMS farms, respectively. Pure infections were less frequent, with *C. parvum* in 13 of 61 and 4 of 27 straw-bedded and RMS farms, respectively, with *C. andersoni* found only in 1 farm using straw bedding.

Eimeria was isolated from 39 of 61 straw-bedded farms and in most RMS farms: in manure (21 of 27), manure solid fraction (24 of 27), RMS before use (22 of 27), and used RMS (22 of 27). The proportion of manure samples from RMS-bedded farms (77.8%) positive for *Eimeria* spp. was not statistically different from that of manure samples from straw-bedded farms (63.9%; $P = 0.20$; chi-squared test). The proportion of manure samples from RMS-bedded farms positive for *E. bovis*

Table 1. Parasitic agents identified in dairies using straw bedding (n = 61) and recycled manure solids bedding (RMS; n = 27)

Parasite	Straw-bedded farms, manure	RMS-bedded farms			
		Manure	Manure solid fraction	RMS before use	Used RMS
Cryptosporidium spp.	30 (49.2%)[a]	23 (85.2%)[b]	22 (81.5%)	23 (85.2%)	26 (92.9%)
Eimeria spp.	39 (63.9%)	21 (77.8%)	24 (88.9%)	22 (81.5%)	22 (81.5%)
E. bovis	13 (21.3%)	10 (37.0%)	5 (18.5%)	5 (18.5%)	4 (14.8%)
E. zuernii	6 (9.8%)	4 (14.8%)	4 (14.8%)	3 (11.1%)	4 (14.8%)
Giardia spp.	—	1 (3.7%)	—	—	—
Moniezia spp.	8 (13.1%)	1 (3.7%)	1 (3.7%)	—	1 (3.7%)
Toxocara spp.	1 (1.6%)	—	—	—	—

[a,b]Values within a row with different superscripts differ ($P < 0.05$).

(37.0%) or *E. zuernii* (14.8%) was not statistically different from that of manure samples from straw-bedded farms (21.3% for *E. bovis*; $P = 0.12$; and 9.8% for *E. zuernii*; $P = 0.50$; Chi-squared test).

Giardia spp. trophozoites or oocysts were isolated from only 1 manure sample recovered from an RMS herd. This may be due to the environmental fragility of this parasite compared with *Cryptosporidium* spp. and *Eimeria* spp. No protozoan parasites were found in any sample derived from bulk tank milk, neither by microscopy analysis nor by molecular methods.

Parasite Survival Through RMS Preparation Procedures

Median egg counts for all parasite species did not differ between manure and any stage of RMS preparation or use (Table 2). Mean *Cryptosporidium* oocyst count (95% CI) per gram of materials across 3 replicates was 4.20 (1.54, 11.50), 2.20 (0.80, 6.04), 3.19 (1.22, 8.35), and 1.95 (0.75, 5.02), for manure, manure solid fraction, RMS before use, and used RMS, respectively. See Table 2 for mean *Eimeria* spp. oocyst count (95% CI) per gram of materials. The count of oocysts of *Cryptosporidium* spp. in manure was not associated with counts in unused RMS ($P = 0.84$). Similarly, counts of *Eimeria* spp. (when considered as a group) in manure were not associated with counts in unused RMS ($P = 0.10$). Counts of *E. bovis* in manure, however, were associated with counts in unused RMS ($P = 0.03$); an increase of 1 oocyst of *E. bovis* in manure resulted in 1.7 times (95% CI: 1.1, 2.8) more oocysts of that pathogen in unused RMS. Counts of *E. zuernii* in manure were not associated with counts in unused RMS ($P = 0.61$).

Parasite Egg Counts in Manure for Herds Housing Cows on RMS Compared with Straw Bedding

Oocyst counts in manure of cows housed on straw bedding and on RMS are described in Table 3. Median estimated *Cryptosporidium* spp. egg count in manure obtained from RMS- and straw-bedded herds was not statistically different ($P = 0.34$). Similarly, no difference was observed between RMS- and straw-bedded herds in median estimated counts of *Eimeria* spp. ($P = 0.76$), nor in counts of *E. bovis* ($P = 0.69$) and *E. zuernii* ($P = 0.53$).

DISCUSSION

As bedding, RMS has many potential benefits, including increased cow comfort and on-site availability, making it a possible alternative to more traditional systems such as straw- or sand-based bedding (Leach et al., 2015). However, it still carries many potential risks to animal and human health. Among these risks,

Table 2. Predicted median egg counts (LSM estimates in number of oocysts per gram of materials) in manure, manure solid fraction after separation, recycled manure solids (RMS) bedding before use, and RMS bedding at the end of the use cycle, for different common bovine digestive tract parasites

Biological material	Estimated median egg count (95% CI) per gram of materials			
	Cryptosporidium spp.	Total *Eimeria* spp.	*Eimeria bovis*[1]	*Eimeria zuernii*
Manure	4.20 (1.54, 11.50)	0.24 (0.08, 0.77)	0.30 (0.08, 0.98)	0.02 (0.00, 0.13)
Manure solid fraction	2.20 (0.80, 6.04)	0.20 (0.07, 0.58)	0.12 (0.03, 0.38)	0.00 (0.00, 0.08)
RMS before use	3.19 (1.22, 8.35)	0.58 (0.18, 1.80)	0.15 (0.05, 0.62)	0.00 (0.00, 0.08)
Used RMS	1.95 (0.75, 5.02)	0.47 (0.15, 1.40)	0.13 (0.03, 0.47)	0.00 (0.00, 0.07)

[1]For the *E. bovis* model, we could not obtain convergence of the generalized negative binomial mixed model, and a generalized negative binomial model had to be used instead, ignoring clustering of observations by herds.

different key pathogens (including bacteria, parasites, and viruses) should be considered in herds that use RMS (Leach et al., 2015). Bacterial populations in RMS have been studied, mainly due to their potential link to clinical mastitis (Leach et al., 2015; Bradley et al., 2018). However, parasite populations in RMS are not well understood, and no report to date has raised any concerns about parasite-related risk when using or working with RMS.

Our results revealed protozoan and metazoan parasites in both types of bedding. Metazoan parasites, such as lungworms and GIN, were less frequent (Table 1), mainly because the L3 stage (infective form) of these parasites is not able to complete its life cycle within confinement-housing conditions (Kumar et al., 2013; Auld and Tinsley, 2015).

Regarding protozoan parasites, our analyses led to the isolation of *Giardia* spp., *Eimeria* spp., and *Cryptosporidium* spp. *Giardia* oocysts were only identified in manure from 1 out of 27 RMS farms, which could be explained by a very low prevalence in Quebec herds, by the age of the studied population (mostly adult cattle, which are less prone to *Giardia* infections), or by the rapid degradation of the parasites in feces (1 week in cattle feces, according to Olson et al., 1999), hindering the detection of the parasite in our samples. Coccidia occur in all breeds of cattle and, although the disease is seen more commonly in calves 3 wk to 6 mo of age, it may occur in yearlings and adults (Keeton and Navarre, 2018). Our analyses found *Eimeria* spp. in most herds, regardless of bedding type (Table 1), which correlates with previous studies conducted in other regions of Canada (Radostits and Stockdale, 1980; Kennedy and Kralka, 1987). Our morphology study revealed 2 highly pathogenic *Eimeria* species, *E. bovis* and *E. zuernii*, which mainly affect calves aged up to 1 yr. Infected calves frequently suffer from severe diarrhea, fever, abdominal pain, occasionally muscular tremors, convulsions, anemia, dehydration, weakness, and anorexia, which leads to impaired performance and sometimes the death of the animal. Asymptomatic cattle frequently act as a source of infection for calves, as fecal material containing oocysts may contaminate feed, water, and soil (Keeton and Navarre, 2018). Consequently, RMS could represent a risk for calves if the oocysts present in the manure are not inactivated during the recycling process. That said, the low median oocyst counts observed in this study (Table 2) suggest that bedding would be a minimal source of potential infection. Our results revealed a significant increase in the number of *Eimeria* oocysts present in RMS before use (Table 2), which could be explained either by contamination by non-cattle coccidia (rodents, birds, etc.) or by recontamination of RMS induced by cows in the stalls.

Cryptosporidium parasites were found in 50% and 85 to 93% of straw-bedded and RMS farms, respectively. This is in agreement with other studies, which have reported prevalence values ranging from 55 to 100% in Canadian herds (Olson et al., 1997; Budu-Amoako et al., 2012a). The fact that *Cryptosporidium* was found in manure in a higher proportion of RMS herds compared with straw-bedded herds could be due to using manure solids as bedding. Indeed, *Cryptosporidium* oocysts are known to be very resistant, even in harsh environments. It is thus very unlikely that RMS preparation methods would lead to any significant reduction in oocyst counts. Nevertheless, the association observed could also be due, in part, to another extraneous factor. For instance, RMS and straw-bedded farms may differ in terms of DHI participation, herd size, or season of sample collection. To investigate whether the obtained result could be solely due to confounding by another unmeasured characteristic, we computed the E-value described by VanderWeele and Ding (2017), using the package EpisensR (VanderWeele and Ding, 2017). This E-value is defined as the minimum strength of association, on the risk ratio scale, that an unmeasured confounder would need to have with both the exposure and the disease to fully explain a specific association. With the current data, a hypothetical confounder would have to increase the risk of cryptosporidiosis by a factor of 4.3 to fully create the observed association. It is thus very unlikely that the observed association of more *Cryptosporidium*

Table 3. Mean, median, and range of oocyst count (per gram of manure) for the most common parasites in manure from straw-bedded cows versus cows bedded on recycled manure solids (RMS)

	Straw-bedded cows			RMS-bedded cows		
Parasite	Mean	Median	Range	Mean	Median	Range
Cryptosporidium spp.	16.83	0.00	0.00–549.17	31.00	1.17	0.00–619.00
Eimeria spp.	0.75	0.00	0.00–19.17	0.90	0.00	0.00–6.00
E. bovis	0.10	0.00	0.00–2.83	0.30	0.00	0.00–3.00
E. zuernii	0.08	0.00	0.00–1.17	0.15	0.00	0.00–2.50

in RMS farms would be due to confounding by an extraneous unmeasured variable, such as herd size or age of the facilities.

Cryptosporidium parvum is typically found in preweaned calves and is responsible for acute diarrhea. Our results isolated *C. parvum* from most farms, which is similar to the range of 40 to 88% reported in previous Canadian studies (Ruest et al., 1998; Trotz-Williams et al., 2005). *Cryptosporidium parvum* has frequently been linked to zoonotic transmission in personnel and professionals such as veterinarians, who work with cattle (Gormley et al., 2011; Pumipuntu and Piratae, 2018). Moreover, evidence suggests an indirect path of zoonotic transmission from cattle to humans mediated by contaminated water sources, as reported in Prince Edward Island (Budu-Amoako et al., 2012b). These 2 routes of transmission could be favored when using RMS that has not tested pathogen-free. We found *C. parvum* oocysts at all stages of RMS preparation, showing that this process has no potential effect on the reduction of the total number of parasites. In this study we did not evaluate infectivity of oocysts. However, environmental resistance of *Cryptosporidium* oocysts, coupled with the fact that a large majority of RMS farms simply extracted a fraction of the liquid and then let the solid fraction mature in a pile or a container for various time periods, without any temperature or time threshold controls, makes it very likely that most oocysts remain infective. This could have 2 major implications. (1) Without having been tested for pathogens, RMS should not be used for calves, given that they are more likely to suffer from *Cryptosporidium* infections. Several studies have shown that age is associated with *Cryptosporidium* infection, with young calves being the population at highest risk of infection (Santín et al., 2004; Maddox-Hyttel et al., 2006; Fayer et al., 2007). (2) Because *C. parvum* can infect humans through fecal-oral contact or airborne dust particles (Balderrama-Carmona et al., 2014) at doses as low as 10 to 30 oocysts (Messner and Berger, 2016), the oocyst counts found in this study suggest a potential risk of zoonotic infection when working with RMS. Better protocol, including a validated composting process that guarantees total inactivation of these parasites, should be implemented on RMS farms.

Cryptosporidium andersoni was detected in 31 and 74% of straw- and RMS-bedded farms, respectively. This species has been described as one of the most frequent species of *Cryptosporidium* infecting cattle in Canada (Budu-Amoako et al., 2012a) and worldwide (Robertson et al., 2014). This parasite infects the gastric gland of the abomasum of older post-weaned calves, yearlings, and adults. *Cryptosporidium andersoni* has been reported to reduce milk production in dairy cows by approximately 13% (Anderson, 1998). Future studies should target this issue and determine whether the presence of *C. andersoni* in RMS farms may cause a decrease in production at both the animal and herd levels. Finally, *C. meleagridis* has a wide host range, including cattle, but mainly infects birds (Gong et al., 2017), which implies possible accidental transmission rather than a real infection—for instance, ingested oocysts that pass intact through the gastrointestinal tract or environmental contamination from free-living wild birds (Sevá et al., 2011).

Raw milk and raw milk products have been reported as the cause of several cases of human cryptosporidiosis, mainly by *C. parvum* (Laberge et al., 1996; Djuretic et al., 1997; Harper et al., 2002). However, this parasite was not retrieved from bulk tank milk in any of the sampled herds. Thus, the possibility of any mammary gland contamination or milk infection during harvesting is hypothesized to be low or nil.

Our transverse study design did not allow determination of the chronological order of occurrence between exposure and outcome. In our case, it is unlikely that many producers with a very high parasitic burden in their herd recently decided to switch to a new bedding (RMS) to control this issue (rather than bedding type being the cause for the parasite burden). First, parasitic burden is very uncommonly investigated in Quebec's dairies. Second, bedding (and, more specifically, RMS) would hardly be considered as a means to control parasitic burden. Finally, we only considered herds having used a given type of bedding for at least 6 mo before measuring the parasitic burden. Another limitation is that RMS was only compared with straw bedding, although straw is the most commonly used bedding in Quebec's dairies.

CONCLUSIONS

This study represents the first attempt to evaluate the parasite burden in farms of the province of Quebec, Canada, using RMS. Despite the limitations discussed, results show that RMS does not seem to have an effect on parasite diversity or frequency compared with straw-based bedding. However, current uncontrolled strategies for preparing RMS do not guarantee the destruction of protozoan parasites, as oocysts are found at every step of RMS preparation and transformation, a process which is not standardized between farms. Considering the relatively high prevalence of *Cryptosporidium* parasites in RMS found in this study, including zoonotic *C. parvum*, further work is needed to evaluate the infectivity of oocysts and quantify the risk of environment-to-animal transmission, especially when RMS is used for calves, as well as the risk RMS poses to farm workers and to water supplies.

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