Bedding and bedding management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk

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ABSTRACT

Mesophilic and thermophilic spore-forming bacteria represent a challenge to the dairy industry, as these bacteria are capable of surviving adverse conditions associated with processing and sanitation and eventually spoil dairy products. The dairy farm environment, including soil, manure, silage, and bedding, has been implicated as a source for spores in raw milk. High levels of spores have previously been isolated from bedding, and different bedding materials have been associated with spore levels in bulk tank (BT) raw milk; however, the effect of different bedding types, bedding management practices, and bedding spore levels on the variance of spore levels in BT raw milk has not been investigated. To this end, farm and bedding management surveys were administered and unused bedding, used bedding, and BT raw milk samples were collected from dairy farms (1 or 2 times per farm) across the United States over 1 yr; the final data set included 182 dairy farms in 18 states. Bedding suspensions and BT raw milk were spore pasteurized (80°C for 12 min), and mesophilic and thermophilic spores were enumerated. Piecewise structural equation modeling analysis was used to determine direct and indirect pathways of association among farm and bedding practices, levels of spores in unused and used bedding, and levels of spores in BT raw milk. Separate models were constructed for mesophilic and thermophilic spore levels. The analyses showed that bedding material had a direct influence on levels of spores in unused and used bedding as well as an indirect association with spore levels in BT raw milk through used bedding spore levels. Specific bedding and farm management practices as well as cow hygiene in the housing area were associated with mesophilic and thermophilic spore levels in unused bedding, used bedding, and BT raw milk. Notably, levels of spores in

used bedding were positively related to those in unused bedding, and used bedding spore levels were positively related to those in BT raw milk. The results of this study increase the understanding of the levels and ecology of mesophilic and thermophilic spores in raw milk, emphasize the possible role of bedding as a source of spores on-farm, and present opportunities for dairy producers to reduce spore levels in BT raw milk.

Key words: spore, raw milk, bedding, management practice

INTRODUCTION

Due to increasingly stringent quality requirements set by national and international customers, the United States dairy industry has been challenged to minimize spore-forming bacteria levels in their products (Gopal et al., 2015). When present as spores, these bacteria are capable of surviving stressful conditions associated with processing raw milk into dairy products. Bacterial spores can survive in dairy products for extensive time periods, and once conditions are favorable for vegetative growth, these bacteria are able to germinate and grow to cause spoilage (Gauvry et al., 2017). Product contamination with spore-forming bacteria is of great concern to the dairy industry across product sectors. Spores isolated from HTST and UHT milk as well as concentrated milk are predominantly *Bacillus* spp. and *Paenibacillus* spp. (Ivy et al., 2012; Kmiha et al., 2017; Martinez et al., 2017). A range of mesophilic and thermophilic spores have been isolated in dairy powders and products manufactured using dairy powders as ingredients, with *Bacillus licheniformis* (able to grow at mesophilic as well as thermophilic temperatures) and *Geobacillus* spp. and *Anoxybacillus* spp. (obligate thermophiles) being the most frequently isolated (Miller et al., 2015b; Sadiq et al., 2016).

In the context of the dairy food chain, mesophilic and thermophilic spores have been found in the farm environment and bulk tank (BT) raw milk as well as in the processing environment and in finished dairy products (Lücking et al., 2013; Watterson et al., 2014; Ortuzar

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et al., 2018). Although there are many opportunities for these bacteria to contaminate product along the dairy continuum, raw milk has been highlighted as one of the principal contamination sources (Huck et al., 2007). Despite developments in processing technologies such as bactofugation and membrane filtration, aimed at removing spores from milk, costs associated with implementation of these technologies may be prohibitive (Brändle et al., 2016). The United States dairy industry is shifting toward a system in which processors monitor levels of spores in incoming raw ingredients (Murphy et al., 2016) and producers are being pressured to react to processor demands to control spores in the raw milk.

The dairy farm environment, including bedding, silage, manure, and soil, has been highlighted as a source of spore-forming bacteria in BT raw milk (Carlin, 2011; Gleeson et al., 2013). Bedding in particular has been implicated as a source of spores in BT raw milk (via contamination of the udder and teats) because lactating cows spend 12 to 14 h/d lying down in direct contact with bedding (Rowbotham and Ruegg, 2015). Previous studies have demonstrated that factors that influence contamination events, persistence, and growth of vegetative bacteria in unused and used bedding materials are complex (Hogan et al., 1989; LeJeune and Kauffman, 2005; Godden et al., 2008), and our understanding of spores in unused and used beddings is limited (Viaene et al., 2017; Bradley et al., 2018). Furthermore, although different farm management practices have been identified as associated with the presence and levels of specific groups of spores (e.g., psychrotolerant, mesophilic, and thermophilic) in BT raw milk (Masiello et al., 2014, 2017; Miller et al., 2015a), there is no consensus on the role of bedding material and bedding management practices in spore contamination of BT raw milk (Miller et al., 2015a; Bradley et al., 2018). Thus, the objectives of this study were (1) to determine spore levels in different unused and used beddings, (2) to determine spore levels in BT raw milk from farms where lactating cows are exposed to different beddings, and (3) to determine associations between different bedding types, bedding management practices, and bedding spore levels and mesophilic and thermophilic spore levels in BT raw milk.

MATERIALS AND METHODS

Farm Enrollment

Members of the Laboratory for Udder Health Veterinary Diagnostic Laboratory (LUH; University of Minnesota, St. Paul), mastitis researchers involved in

the NE1048 Multi-State Mastitis Research Project, and members of the American Association of Bovine Practitioners recruited farms across the United States according to defined criteria. Participating farms were required to (1) be on a regular DHIA testing program (≥ 9 tests/yr), (2) use the same kind of bedding for all lactating cow groups, (3) be willing and able to record all lactating cow clinical mastitis events, and (4) allow the sampler to visit twice to administer a farm management survey, score cow hygiene in the housing area, and collect bedding and BT raw milk samples. A total of 190 farms in 18 states in the United States were enrolled.

Survey of Management Practices

A total of 62 veterinary and university extension professionals were recruited by investigators at the University of Minnesota as samplers and were assigned to farms based on proximity. A farm management survey was prepared by the LUH; this survey included questions regarding herd characteristics, housing systems for lactating cows, type and management of bedding, and milking procedures (Table 1). The farm management survey was accompanied by a cow hygiene scoring form (adapted from the University of Wisconsin Udder Hygiene Scoring Chart; Dairy Team Extension, 2002) with instructions for samplers for assessing the cleanliness of the udder and hind legs of a total of 20 cows in the housing area while collecting bedding samples. Hygiene scores were assigned using a 4-point scale (1 = the surface area was free of dirt; 2 = 2–10% of the surface area was covered; 3 = 10–30% of the surface area was covered; 4 = >30% of the surface area was covered). Farms were visited by an assigned sampler twice between January 6, 2016, and February 3, 2017. At each sampling, a farm management survey was administered, cow hygiene in the housing area was assessed, and unused bedding, used bedding, and BT raw milk samples were collected.

Sample Collection

Samplers were provided with printed instructions by the LUH for sample collection and shipping. Briefly, samplers wore clean gloves to collect 15 handfuls of bedding (either unused or used), which were then commingled in clean buckets and subsequently divided between 2 plastic resealable bags. Unused bedding samples were collected at farms from the bedding storage area (e.g., new sand storage pile). For dry lot (DL) farms that were not adding new bedding (e.g., in summer), samplers instead collected unused bedding from

Table 1. Summary of farm and bedding management practices data collected from surveys administered during sample collection and selected for inclusion in structural equation modeling analyses

Factor	Description
Herd health	(1) Number of clinical cases of mastitis in 30 d before sample collection per number of lactating cows
Cow hygiene	(1) Cow hygiene score in housing area; (2) tails are docked or only switches on tails are trimmed or neither; (3) udder hair is removed by clipping or singeing at least once per lactation
Milking routine	(1) Number of milkings per day; (2) milking units washed or sprayed off between uses; (3) type of premilking teat dip used; (4) type of postmilking teat dip used; (5) forestrip teats premilking; (6) use an automatic teat scrubber
Housing	(1) Type of lactating cow housing; (2) lactating cow access to outside paddock or exercise yard; (3) number of lactating cows per number of stalls for freestall or tiestall housing; (4) stall body length from neck rail or brisket board to back of curb for freestall or tiestall housing
Bedding material	(1) Bedding material is organic or inorganic; (2) type of bedding material for lactating cows
Unused bedding management	(1) New sand washed before purchase; (2) method of recycling reclaimed sand; (3) reclaimed sand stored in the open or under cover; (4) reclaim sand year-round or only for part of the year; (5) time in storage from recapturing sand or manure solids to reusing in stalls; (6) manure solids are pressed before use; (7) manure solids are mechanically dried before use
Used bedding management	(1) Bedding conditioner added to stalls; (2) frequency of scraping manure from back of stall surface or removal from yard or pack; (3) frequency of adding new bedding material to the stalls or bedded pack or yard area; (4) frequency of scraping alleyways behind the stalls; (5) how often the dry lot or bedded pack surface is raked or tilled; (6) number of times per year that used bedding is completely removed and then replaced with unused bedding in deep-bedded freestalls, deep-bedded tiestalls, or bedded pack or dry lot

pens posttilling. Used bedding samples were collected at freestall (**FS**) and tiestall (**TS**) farms from the middle back third of 15 stalls, whereas samples were collected at bedded pack (**BP**) and DL farms from different areas of pens pretilling. For farms with pens (i.e., FS, BP, and DL), samples were collected from up to a maximum of 5 pens (3 bedding samples/pen $\times$ 5 pens = 15 bedding samples). Bedding samples were stored frozen on-farm before transport to the LUH.

Within 7 d of bedding sample collection, BT raw milk samples were collected in duplicate daily for 3 consecutive days and were collected either from the on-farm BT or from the tanker truck at the processing facility (for farms that directly load onto tankers). Following previously described procedures (Servello et al., 2004), tanks were agitated for at least 2 min before collection of approximately 30 mL with a sterile dipper or syringe. Milk samples were transferred to sterile 60-mL polypropylene terephthalate vials and were immediately frozen.

Bedding and BT raw milk samples were shipped to the LUH, where they were then stored at -20°C until samples were shipped overnight to the Milk Quality Improvement Program (Cornell University, Ithaca, NY) in an extruded polystyrene foam cooler packed with ice packs. Sample sets were rejected if received $>6^{\circ}\text{C}$ or if sample sets were missing or had insufficient amounts of milk, unused bedding, or used bedding. Samples were stored at -20°C until further analysis.

Microbiological Evaluation of Farm Samples

Samples were allowed to thaw at 6°C for approximately 12 h before processing. Bedding samples were

transferred to a filter stomacher bag and suspended in a 1:10 (wt/wt) ratio in Butterfield's buffer and homogenized for 60 s at 260 rpm in a Stomacher 400 Circulator (Seward Ltd., Worthing, UK); bedding samples with sharp objects (e.g., rocks) were manually agitated for 60 s. A 20-mL aliquot of each bedding suspension was transferred into an individual sterile screw-capped glass tube, which was stored on ice until heat treatment. Individual BT raw milk samples were aseptically transferred into a sterile 10-oz. (300 mL) polypropylene terephthalate vial and shaken 25 times to be completed within 7 s according to Frank and Yousef (2004) before a 20-mL aliquot was transferred into an individual sterile screw-capped glass tube, which was stored on ice until heat treatment.

Aliquots of each bedding suspension and BT raw milk sample were spore pasteurized at 80°C for 12 min, followed by rapid cooling to $<6^{\circ}\text{C}$ (Kent et al., 2016). Consistent with previous studies (Kent et al., 2016; Martin et al., 2019), 50- μL aliquots of spore-pasteurized unused bedding and used bedding suspensions were spiral plated (Autoplate 5000, Advanced Instruments Inc., Norwood, MA) in duplicate onto brain heart infusion (**BHI**) agar (BD Diagnostics, Franklin Lakes, NJ), and 1-mL aliquots of spore-pasteurized milk samples were pour plated in duplicate in BHI followed by incubation at 32°C for 48 h for mesophilic spore count (**MSC**) or 55°C for 48 h for thermophilic spore count (**TSC**), followed by enumeration.

Bacterial Isolate Collection

Morphologically distinct colonies were selected from all sample types and streaked onto BHI agar plates.

Pure cultures were grown overnight in BHI broth at 32°C and then preserved in 15% glycerol and stored in duplicate at -80°C. A total of 5,263 isolates were collected over the duration of the study. Isolate information is available through Food Microbe Tracker (<http://www.foodmicrobetracker.com>).

Data Clean-Up

Survey data and spore count data were managed separately in Excel (version 13.1.1, Microsoft Corp., Redmond, WA). Before data analysis, survey questions and responses were reviewed with factors removed due to the following reasons: (1) not applicable given the study purposes (e.g., type of dry cow housing), (2) misleading question phrasing (e.g., How long has the current bedding system been in use?), and (3) missing responses. Selected survey data (Table 1) and spore count data were joined and cleaned in OpenRefine (version 2.7; <https://openrefine.org>).

Data Analyses

All analyses were performed in the R Statistical Programming Environment (R Core Team, 2018). Spore count data were log-transformed to achieve an approximate normal distribution. To calculate arithmetic means, values below the detection limit (i.e., $<2.00 \log_{10}$ cfu/g for unused and used bedding; $<-0.30 \log_{10}$ cfu/mL for BT raw milk) were substituted with values equal to half of the detection limit. For statistical model analyses, a constant value of 1 was added to unused bedding and BT raw milk spore count data before applying the log-transformation.

A priori model was hypothesized, assuming that changes of spore count in unused bedding, spore count in used bedding, and bedding and farm management practices affect the variations of BT raw milk spore count. We constructed 3 individual linear mixed-effects models (**LMM-I**, **LMM-II**, and **LMM-III**), each of which included hypothesized relationships between a response variable and a set of predictors as fixed effects, and farm ID as a random effect on the intercept. Each linear mixed-effects model was fit using the lmer function in the lme4 package (Bates et al., 2015), and the piecewise structural equation model, combining models for each response variable, was fit using the piecewiseSEM package in R (Lefcheck, 2016). The response variables were (1) unused bedding spore count for LMM-I, (2) used bedding spore count for LMM-II, and (3) BT milk spore count for LMM-III. In some cases, a variable included as a response in one linear mixed-effects model was also included as a predictor in

a different linear mixed-effects model (e.g., used bedding spore count). The predictors included were (1) for LMM-I bedding type and material and specific bedding management practices applicable for unused bedding; (2) for LMM-II unused bedding spore count, bedding type and material, specific farm, and bedding management practices applicable for used bedding; and (3) for LMM-III used bedding spore count, bedding type and material, and specific farm management practices applicable for BT raw milk. To address multicollinearity, variance inflation factors were investigated and brought into an acceptable range (Zuur et al., 2010). Missing paths were determined, and models were adjusted (Lefcheck, 2016). Overall model adequacy was determined by Shipley's test of directed separation and the small sample size-corrected version of Akaike's information criterion (Shipley, 2013; Lefcheck, 2016). Indirect effects were predicted and then evaluated using the Sobel test (Kline, 2005). Unstandardized coefficients (left in their original units), their associated standard error, and *P*-values were generated from the initial structural equation model; subsequently, standardized coefficients and their associated standard error were generated after rerunning the structural equation model with scale standardization (scaled by mean and variance) to allow comparisons across variables of varying units (Lefcheck, 2016). Individual direct and indirect paths were considered significant if $P \leq 0.05$. Data analyses were performed separately for mesophilic and thermophilic spore levels. Data and code to reproduce the results are available on GitHub (https://github.com/sim39/JDS_BeddingManagement.git).

RESULTS

Bedding Type and Management Practices Vary by Farm

The majority of the 190 farms that participated in this study were surveyed and sampled twice (176/190; 93%). After accounting for sample sets and survey data that met defined inclusion criteria, 182 farms were ultimately retained in the data set, including data from 2 samplings for 110 farms and 1 sampling for 72 farms (i.e., 292 total farm samplings). Farm sampling data were removed when (1) cow hygiene was not assessed ($n = 1$), (2) the sample collection procedure was not followed ($n = 8$), (3) shipped samples arrived $> 6^{\circ}\text{C}$ and no replacement samples were obtained ($n = 61$), or (4) a laboratory error occurred ($n = 4$).

All 182 farms retained in the data set were conventionally operated dairies located in different regions of the United States, including the Northeast (ME,

NY, VT; 16 farms, 26 farm samplings), Midwest (IA, IN, MI, MN, MO, OH, WI; 122 farms, 202 farm samplings), South (FL, GA, TX; 8 farms, 15 farm samplings), and West (CA, ID, OR, SD, WA; 36 farms, 49 farm samplings; Table 2). Farm management practices on retained farms were varied and included a range of housing types (i.e., FS, TS, BP, and DL), bedding types [i.e., new sand (**NS**), reclaimed sand (**RS**), limestone (**LS**), manure solids (**MNS**), shavings or sawdust (**SH**), straw (**ST**), and other organic bedding (**OO**)], and bedding management practices (Table 2). Herd sizes ranged from 74 to 21,830 cows, with a mean size of 1,910 cows and a median of 860. Cow breeds included Holstein (73%), Jersey or Brown Swiss (6%), and multiple or mixed breeds (21%). The number of lactating cows per farm ranged from 35 to 9,650, with a mean of 879 lactating cows and a median of 408. Milk production across farms ranged from 7,127 to 15,649 kg/cow per year (mean of $11,694 \pm 1,688$ kg). Farms with a TS milking system (7%) milked cows 2× per day, whereas farms with a milking parlor (93%) milked cows between 2× and 4× daily (51% at 2×, 48% at 3×, and 1% at 4×). In the 30 d preceding bedding sample collection, the percentage of clinical mastitis events in the lactating herd ranged from 0 to 0.23% (mean of $0.04 \pm 0.03\%$). Average herd SCC across farms ranged from 55,000 to 837,000 cells/mL (geometric mean of 193,994 cells/mL).

Farms across all regions reported housing lactating cows in FS (88% of farm samplings); BP farms (3%) were represented in the Midwest and South, TS farms (7%) were represented only in the Midwest and Northeast, and DL farms (2%) were represented only in the South and West (Table 2). A similar proportion of FS farms used organic and inorganic bedding materials (47 and 53%, respectively). Among non-FS farms ($n = 36$), 94% used organic bedding materials; DL farms ($n = 6$) used only MNS (100%), whereas BP farms ($n = 9$) used either MNS (22%) or SH (78%; Table 2).

Mesophilic and Thermophilic Spore Levels Vary by Bedding Material

After sample processing, individual data points were removed for MSC analysis ($n = 2$) and TSC analysis ($n = 2$) due to swarming of spore-forming bacteria that prevented reliable enumeration; data points were also removed for TSC analysis due to laboratory error ($n = 2$). As such, 286 farm samplings were represented in both the MSC and TSC analyses, 2 farm samplings were represented only in the MSC analysis, and 4 farm samplings were represented only in the TSC analysis. Altogether, data from 290 farm samplings (182 farms) were used for MSC analysis and data from 288 farm

Table 2. Farm characteristics of the 182 enrolled farms in different regions of the United States

Region	No. of farms	No. of farm samplings	Mean herd size (no. of cows)	Milk production ¹ (kg/yr)	Herd geometric mean SCC ² ($\times 1,000$ cells/mL)	No. of farm samplings per housing type ³ (%)				No. of farm samplings per bedding type ³ (%)						
						Freestall	Tiestall	Bedded pack	Dry lot	New sand	Reclaimed sand	Limestone	Shavings/sawdust	Manure solids	Straw	Other
Midwest	122	202	1,214	11,886 $\pm$ 1,671	205	176 (87)	19 (9)	7 (4)	—	69 (34)	35 (17)	2 (1)	47 (23)	24 (12)	21 (11)	4 (2)
Northeast	16	26	1,319	11,246 $\pm$ 1,292	160	24 (92)	2 (8)	—	—	8 (31)	3 (11)	—	9 (35)	2 (8)	—	4 (15)
South	8	15	3,937	10,817 $\pm$ 1,475	152	12 (80)	—	2 (13)	1 (7)	2 (13)	7 (47)	—	—	6 (40)	—	—
West	36	49	4,474	11,411 $\pm$ 1,870	183	44 (90)	—	—	5 (10)	5 (10)	7 (15)	—	4 (8)	27 (55)	5 (10)	1 (2)
All	182	292	1,910	11,694 $\pm$ 1,688	194	256 (88)	21 (7)	9 (3)	6 (2)	84 (29)	52 (18)	2 (1)	60 (20)	59 (20)	26 (9)	9 (3)

¹Mean $\pm$ SD.

²Not reported for a total of 15 samplings, including Midwest ($n = 5$), Northeast ($n = 2$), and West ($n = 8$).

³Dash indicates that no samples were tested from this group.

samplings (180 farms) were used for TSC analysis. The MSC for unused bedding samples ranged from below the detection limit (i.e., $<2.00 \log_{10}$ cfu/g) to $8.18 \log_{10}$ cfu/g (mean of $4.15 \pm 1.51 \log_{10}$ cfu/g), and TSC ranged from <2.00 to $7.42 \log_{10}$ cfu/g (mean of $3.49 \pm 1.43 \log_{10}$ cfu/g). Among all unused bedding samples tested, 7% (19/290) had MSC below the detection limit ($<2.00 \log_{10}$ cfu/g) and 13% (37/288) had TSC below the detection limit ($<2.00 \log_{10}$ cfu/g). All 292 used bedding samples tested had detectable levels of both mesophilic and thermophilic spores. Used bedding MSC ranged from 2.48 to $8.17 \log_{10}$ cfu/g (mean of $5.53 \pm 0.94 \log_{10}$ cfu/g), and TSC ranged from 2.00 to $8.11 \log_{10}$ cfu/g (mean of $4.56 \pm 1.17 \log_{10}$ cfu/g).

For both used and unused bedding, mean MSC and TSC across all 152 organic bedding samples were higher (see Table 3) compared with the respective means across all 138 inorganic bedding samples. In addition, MSC and TSC were typically higher for the different individual organic bedding materials (i.e., MNS, SH, ST, and OO) compared with inorganic bedding materials (i.e., NS, RS, and LS; Table 3). Manure solids showed the highest MSC and TSC for unused bedding, generally more than 1 log higher than any other materials except the OO category (Table 3). For used bedding, MNS also showed the highest mean MSC; used bedding mean TSC was highest for OO ($5.75 \pm 1.06 \log_{10}$ cfu/g) followed by MNS ($5.45 \pm 0.97 \log_{10}$ cfu/g). Except for LS, which was represented by only 2 samples, NS consistently showed the lowest unused MSC and TSC,

whereas NS and RS showed similar MSC and TSC for used bedding (Table 3).

Spores Were Detected in the Majority of BT Raw Milk Samples

Among all BT raw milk samples tested, 85% (246/290) had MSC above the detection limit and 85% (245/288) had TSC above the detection limit ($\geq -0.30 \log_{10}$ cfu/mL for both TSC and MSC). Bulk tank raw milk MSC ranged from below the detection limit to $1.76 \log_{10}$ cfu/mL (mean of $0.26 \pm 0.56 \log_{10}$ cfu/mL; Supplemental Figure S1, <https://doi.org/10.3168/jds.2018-16022>), and TSC ranged from below the detection limit to $1.50 \log_{10}$ cfu/mL (mean of $0.26 \pm 0.52 \log_{10}$ cfu/mL; Supplemental Figure S2, <https://doi.org/10.3168/jds.2018-16022>). Notably, 55% (24/44) of the BT raw milk samples with less than detectable levels of mesophilic spores had detectable levels of thermophilic spores, and 53% (23/43) of the BT raw milk samples with less than detectable levels of thermophilic spores had detectable levels of mesophilic spores.

Mesophilic Spore Levels in BT Raw Milk Are Influenced by Spore Levels in Unused and Used Bedding Materials

Standardization of model coefficients allows for the comparison of the relative effects of factors across re-

Table 3. Average spore counts for bedding and bulk tank raw milk samples collected across the United States between January 6, 2016, and February 3, 2017¹

Bedding type and material	No. of farms ²	No. of farm samplings ^{3,4}	MSC ⁵ ($\log_{10}$ cfu/g)		TSC ⁵ ($\log_{10}$ cfu/g)	
			Unused bedding	Used bedding	Unused bedding	Used bedding
Inorganic bedding						
New sand	49	84	3.06 ± 0.84	5.03 ± 0.73	2.36 ± 0.61	3.96 ± 0.92
Reclaimed sand	34	52	4.50 ± 0.79	5.00 ± 0.69	3.74 ± 0.91	4.08 ± 1.00
Limestone	1	2	2.15 ± 0.64	4.84 ± 0.59	ND ⁶	3.62 ± 0.40
Total	84	138	3.59 ± 1.09	5.02 ± 0.71	2.87 ± 1.00	4.00 ± 0.94
Organic bedding						
Shavings/sawdust	38	60	3.21 ± 0.96	5.57 ± 0.83	2.79 ± 0.77	4.67 ± 1.15
Manure solids	43	58	6.03 ± 0.94	6.44 ± 0.82	5.41 ± 0.85	5.45 ± 0.97
Straw	17	25	4.61 ± 1.38	5.88 ± 0.72	3.56 ± 1.22	4.88 ± 1.02
Other organic ⁷	7	9	5.72 ± 1.56	6.19 ± 0.57	5.34 ± 1.37	5.75 ± 1.06
Total	105	152	4.67 ± 1.66	5.99 ± 0.88	4.06 ± 1.52	5.06 ± 1.12

¹MSC = mesophilic spore count; TSC = thermophilic spore count.

²For TSC, new sand = 48 farms and manure solids = 42 farms.

³For TSC, new sand = 83 farm samplings and manure solids = 57 farm samplings.

⁴A total of 7 farms switched bedding materials between samplings.

⁵Mean $\pm$ SD.

⁶Below the detection limit ($<2.00 \log_{10}$ cfu/g).

⁷Other organic bedding materials sampled included casella organics (n = 3), corn stalks (n = 1), rice hulls (n = 1), or mixes including paper + oat hulls (n = 1), paper fiber + wood ash + sawdust (n = 1), manure solids + straw (n = 1), and shavings + paper (n = 1).

sponses. Standardized coefficients (β) are either positive or negative, which can be interpreted as a factor having a positive effect (associated with higher spore levels) or negative effect (associated with lower spore levels) on the response. The effects of categorical factors (as well as continuous factors nested within categorical factors) on the response are reported compared with a defined reference (e.g., the effect of organic bedding on BT raw milk MSC is reported compared with that of inorganic bedding); meanwhile, continuous factors not nested within a categorical variable (e.g., number of milkings per day) do not have a reference. The model also estimates unstandardized coefficients and standard error, which can be interpreted for their predicted influence on the response in the units of the factor (Supplemental Tables S1–S4, <https://doi.org/10.3168/jds.2018-16022>). Given that standardized coefficients allow us to compare relative effect sizes of all factors in the model on the response, we discuss only standardized coefficients [see Supplemental Tables S1–S4 for unstandardized and standardized coefficients, standard errors, and significance tests (P -values) for all evaluated direct and indirect fixed effects].

The structural equation model for mesophilic spore levels identified 5 factors that were directly associated with unused bedding MSC, including 4 factors that were positively associated and 1 factor that was negatively associated with unused bedding MSC. Manure solids bedding had the greatest effect on unused bedding MSC, with a positive β of 0.770, which is indicated in Figure 1 with a thick solid black arrow extending from the “manure solids bedding” box to the “unused bedding” box. Similarly, among organic bedding materials, ST ($\beta = 0.337$) and OO ($\beta = 0.299$) were also positively associated with unused bedding MSC compared with SH (Figure 1; thin solid black arrows). Among inorganic bedding materials, RS was positively associated with unused bedding MSC compared with NS ($\beta = 0.407$; Figure 1). The frequency of unused bedding addition to TS housing, compared with FS housing, was negatively associated with unused bedding MSC ($\beta = -0.120$; Figure 1; thin solid red arrow).

Three factors were directly positively associated with used bedding MSC. Organic bedding type had the greatest effect on used bedding spore count ($\beta = 0.329$; Figure 1). Additionally, among organic bedding materials, MNS was positively associated with used bedding MSC compared with SH ($\beta = 0.234$; Figure 1). Notably, unused bedding MSC was positively associated with used bedding MSC ($\beta = 0.157$; Figure 1).

Three organic bedding materials (MNS, ST, OO) were indirectly positively associated with used bedding

MSC, mediated through unused bedding MSC; this relationship is indicated in Figure 1 by thin dashed black arrows that extend from the factor (e.g., MNS) through the “unused bedding” box to the “used bedding” box. Standardized coefficients for MNS, ST, and OO were 0.121, 0.053, and 0.047, respectively (Figure 1).

Finally, 3 factors were directly associated with BT raw milk MSC. Used bedding MSC had the greatest positive effect on BT raw milk spore count ($\beta = 0.147$; Figure 1). The proportion of cows scored (total $n = 20$) with a hygiene score in the housing area of 3 (compared with a score of 1) was also positively associated with BT raw milk MSC ($\beta = 0.145$; Figure 1). Additionally, among housing types, BP was positively associated with BT raw milk MSC compared with FS ($\beta = 0.133$; Figure 1).

Thermophilic Spore Levels in BT Raw Milk Are Influenced by Certain Farm Management Practices and Spore Levels in Unused and Used Bedding Materials

Factors that were directly associated with unused bedding TSC included MNS, ST, OO, RS, and LS bedding materials as well as the frequency of adding unused bedding to stalls or pens. The MNS bedding, compared with SH, was positively associated with unused bedding TSC and had the greatest effect among factors included in the structural equation model ($\beta = 0.695$; Figure 2). Additionally, among organic bedding materials, ST and OO were also positively associated with unused bedding TSC compared with SH ($\beta = 0.211$ and 0.277 , respectively; Figure 2). Among inorganic bedding materials, RS was positively associated and LS was negatively associated with unused bedding TSC compared with NS ($\beta = 0.522$ and -0.092 , respectively; Figure 2). More frequent addition of unused bedding to TS and DL housing was also associated with lower TSC compared with FS housing ($\beta = -0.147$ and -0.091 , respectively; Figure 2).

Seven factors were directly associated with used bedding TSC. Organic bedding type had the greatest positive effect on used bedding TSC ($\beta = 0.324$; Figure 2). Additionally, among organic bedding materials, ST was positively associated with used bedding TSC compared with SH ($\beta = 0.184$; Figure 2). Notably, unused bedding TSC was positively associated with used bedding TSC ($\beta = 0.169$; Figure 2). Several farm management factors were associated with used bedding TSC, including (1) the frequency of removing manure from pens at farms with BP housing (positively associated with TSC compared with farms with FS housing; $\beta = 0.199$), (2) the frequency of unused bedding addition to BP

housing (negatively associated with used bedding TSC compared with FS housing; $\beta = -0.273$), and (3) the frequency of raking or tilling the bedding surface at farms with DL and BP housing (negatively associated with used bedding TSC; $\beta = -0.275$; Figure 2). Furthermore, BP was associated with used bedding TSC and had the greatest negative effect on used bedding TSC ($\beta = -0.326$); FS was used as a baseline for these comparisons (Figure 2).

Additionally, 3 factors were indirectly associated with used bedding TSC, mediated through unused bedding TSC (Figure 2). Among inorganic bedding materials, RS was indirectly positively associated with used bedding TSC compared with NS ($\beta = 0.088$; Figure 2). Additionally, among organic bedding materials, MNS and OO were indirectly positively associated with used bedding TSC compared with SH ($\beta = 0.117$ and 0.047 , respectively; Figure 2).

Figure 1. Structural equation model of significant effects ($P \leq 0.05$) on mesophilic spore levels in unused bedding, used bedding, and bulk tank raw milk. The width of arrows is proportional to the magnitude of scale-standardized coefficients (β), which are provided on the associated arrows. Black arrows are positive coefficients, and red arrows are negative coefficients. Solid arrows indicate direct effects, and dashed arrows indicate indirect effects. The symbol “=” indicates a particular level of a factor, and the symbol “:” indicates nesting. For each categorical factor and each continuous factor nested within a categorical factor, standardized coefficients are relative to a reference. Numbers in the bottom-left corner of factor boxes correspond to the following references: (1) Bedding type = Inorganic, (2) Bedding type = Inorganic: Bedding material = New sand, (3) Bedding type = Organic: Bedding material = Shavings/sawdust, (4) Housing type = Freestalls, and (5) Proportion of cows scored (total $n = 20$) with hygiene score = 1. Blue boxes indicate response variables. Marginal (R_m^2) and conditional (R_c^2) R-squared are shown for all 3 models (i.e., unused bedding, used bedding, and bulk tank raw milk). Nonsignificant relationships ($P > 0.05$) have been omitted for clarity. Unstandardized and standardized coefficients, standard errors, and significance tests (P -values) for all evaluated direct and indirect fixed effects are provided in Supplemental Tables S1 and S2 (<https://doi.org/10.3168/jds.2018-16022>), respectively.

Three factors were identified as having direct associations with BT raw milk TSC, with used bedding TSC having the greatest effect and being positively associated with BT raw milk TSC ($\beta = 0.209$; Figure 2). The proportion of cows scored (total $n = 20$) with a hygiene score in the housing area of 3 (compared with a score of 1) was also positively associated with BT raw milk TSC ($\beta = 0.201$; Figure 2). Additionally, the practice of removing udder hair by clipping or singeing at least

once per lactation was negatively associated with BT raw milk TSC compared with farms that did not follow this practice ($\beta = -0.151$; Figure 2).

Furthermore, 2 factors were indirectly associated with BT raw milk TSC, mediated through used bedding TSC (Figure 2). Organic bedding type was indirectly positively associated with BT raw milk TSC compared with inorganic bedding type ($\beta = 0.068$; Figure 2). The frequency of unused bedding addition to BP housing

Figure 2. Structural equation model of significant effects ($P \leq 0.05$) on thermophilic spore levels in unused bedding, used bedding, and bulk tank raw milk. The width of arrows is proportional to the magnitude of scale-standardized coefficients (β), which are provided on the associated arrows. Black arrows are positive coefficients, and red arrows are negative coefficients. Solid arrows indicate direct effects, and dashed arrows indicate indirect effects. The symbol “=” indicates a particular level of a factor, and the symbol “:” indicates nesting. For each categorical factor and each continuous factor nested within a categorical factor, standardized coefficients are relative to a reference. Numbers in the bottom-left corner of factor boxes correspond to the following references: (1) Bedding type = Inorganic, (2) Bedding type = Inorganic: Bedding material = New sand, (3) Bedding type = Organic: Bedding material = Shavings/sawdust, (4) Housing type = Freestalls, (5) Udder hair is removed at least once per lactation = no, and (6) Proportion of cows scored (total $n = 20$) with hygiene score = 1. Blue boxes indicate response variables. Marginal (R_m^2) and conditional (R_c^2) R-squared are shown for all 3 models (i.e., unused bedding, used bedding, and bulk tank raw milk). Nonsignificant relationships ($P > 0.05$) have been omitted for clarity. Unstandardized coefficients and standard errors, and significance tests (P -values) for all evaluated direct and indirect fixed effects are provided in Supplemental Tables S3 and S4 (<https://doi.org/10.3168/jds.2018-16022>), respectively.

was indirectly negatively associated with BT raw milk TSC compared with FS housing ($\beta = -0.057$; Figure 2).

DISCUSSION

This study provides a baseline for mesophilic and thermophilic spore levels in different unused and used bedding materials as well as BT raw milk on conventional dairy farms across the United States. Results indicate that used bedding spore levels increase with unused bedding spore levels, and in turn, an increase in used bedding spore levels results in an increase in BT milk spore levels. Further, several intrinsic bedding factors (e.g., bedding material) and bedding management practices (e.g., frequency of adding unused bedding to stalls) were both directly and indirectly associated with spore counts in unused bedding, used bedding, and BT raw milk. These findings improve the understanding of factors affecting spore transmission on dairy farms and suggest that producers who are concerned with BT raw milk spore levels should consider optimizing their bedding programs to reduce spore levels in BT raw milk.

Bedding Material Directly Influences Spore Levels in Unused and Used Bedding and May Indirectly Influence Spore Levels in BT Raw Milk

Bedding type (i.e., organic vs. inorganic) and specific bedding material (e.g., MNS) were identified as being associated with mesophilic and thermophilic spore levels in unused and used beddings. Initial spore levels in unused bedding are likely influenced by several external and intrinsic factors, including (1) spore levels in inputs used to formulate bedding materials; (2) contamination during on-farm processing and storage; and (3) germination, growth, and sporulation of spore-forming bacteria during bedding storage (which depends on intrinsic factors such as nutrient availability and water activity). For example, our finding that organic bedding materials including MNS, ST, and OO were associated with higher spore levels in unused bedding compared with SH may be explained by differences in spore contamination levels in inputs used for bedding materials. Consistent with this, a study by Viaene et al. (2017) showed that fresh manure had the highest concentration of thermophilic spores when different potential bedding material inputs (mechanically separated MNS, mechanically separated MNS with straw and grass, and fresh manure) were tested (Viaene et al., 2017). Another study also supports that manure, the primary material for MNS bedding, can have high levels of spores (e.g., mean MSC and TSC of 5.87 and 5.22

$\log_{10}$ cfu/g, respectively; Martin et al., 2019). Soil also has been shown to contain high levels of spores (mean MSC and TSC of 5.68 and 4.61 $\log_{10}$ cfu/g, respectively; Martin et al., 2019) and hence may contaminate materials such as farm crops or by-products eventually used for bedding materials, including ST and most of the OO bedding materials. In contrast, bedding materials such as SH, which were found here to have low levels of spores, are by-products of wood processing and would likely have little direct contact with a major source of spores such as soil. Spores in unused bedding may also be attributed to contamination events during on-farm bedding processing or storage. For example, producing RS bedding involves combining sand-laden manure with recycled water followed by either passive or mechanical solid-liquid separation (Aguirre-Villegas et al., 2014). Because manure and recycled water may already be contaminated with spores, this process may lead to increased spore levels in RS bedding. In addition to persistence of spores originating from primary sourcing material or introduced through processing, differences in unused bedding spore levels may be related to germination, growth, and sporulation of spore-forming bacteria during preuse bedding storage. Although some materials (e.g., NS) may not exhibit favorable conditions required for growth (e.g., lack of available nutrients), other materials (e.g., MNS) would likely provide a suitable environment for growth. For example, Viaene et al. (2017) found that after 61 d of composting, thermophilic spore levels increased in different manure-associated inputs (e.g., mechanically separated MNS) from 0.4 to 1.4 $\log_{10}$ cfu/g, which suggests that processing of bedding and potentially storage of bedding may increase thermophilic spore levels (Viaene et al., 2017). Moisture content of bedding in particular likely has an effect on spore-forming bacteria in unused bedding; therefore, future studies may include an assessment of moisture content alongside spore levels to provide further explanation for observed differences in spore levels among unused bedding materials. Although growth and resporulation of spore-forming bacteria in bedding have not been directly investigated, several studies have suggested that bedding properties such as particle size, absorbency, available OM and nutrients, DM content, water activity, pH, temperature, and presence of substances toxic to bacteria may influence growth of environmental mastitis-causing bacteria in bedding (Hogan et al., 1989; Zdanowicz et al., 2004; Kristula et al., 2005; LeJeune and Kauffman, 2005; Godden et al., 2008). For example, Godden et al. (2008) identified bedding pH as being positively associated with growth of *Enterococcus faecium* and bedding pH as well as bedding total C (%) as being positively associated with growth of *Klebsiella*

pneumonia. However, after including bedding material as a factor in their analyses, the previously identified bedding biochemical properties were no longer significant. This suggests that selection of specific bedding materials that provide limited support for bacterial growth represents a more valuable control strategy than attempts to try to identify specific bedding lots or supplies with biochemical properties that may be less supportive of microbial growth.

In addition to identifying specific inorganic and organic bedding materials associated with spores in unused bedding, our study identified (1) MNS as being associated with higher used bedding MSC compared with SH and (2) ST as being associated with higher used bedding TSC compared with SH. Similar to unused bedding, these findings may be explained by differences in the ability of different bedding materials, when in use, to provide a suitable environment for growth. A previous study investigating *Bacillus cereus* concentrations in different used bedding materials also found that organic bedding materials, including ST and SH, had higher levels of spores than sand, which is an inorganic bedding material (Magnusson et al., 2007a). A later report suggested that higher levels of spores in used organic bedding compared with inorganic bedding may be attributed to differences in OM and that, in general, spore levels in used bedding materials are influenced by pH, temperature, and the presence of manure and urine (Gauvry et al., 2017).

Our study also identified organic bedding as being indirectly associated with higher TSC in BT raw milk compared with inorganic bedding, mediated through used bedding TSC ($P = 0.0146$). This finding is reasonable given previous understanding of on-farm spore transmission (Vissers et al., 2007a; Carlin, 2011) and provides insight into a potential mechanism by which bedding type influences bacteria levels in BT raw milk (via increasing spore levels in used bedding). Furthermore, this finding is consistent with a previous study that identified a positive association between *Bacillus cereus* spore levels in BT raw milk and spore levels in used bedding (Magnusson et al., 2007a). Additionally, although the influence of bedding type (i.e., whether bedding material is organic or inorganic) had not been previously examined, this finding is also consistent with previous reports identifying certain bedding materials as being associated with BT raw milk spore levels (Miller et al., 2015a; Bradley et al., 2018). Despite the observed significant indirect relationship between organic bedding and TSC levels in BT raw milk, this relationship was not identified as significant ($P = 0.0639$) for MSC. However, because this value could be considered as marginally significant, further investigation into this relationship may be valuable. However, overall,

our data suggest that use of organic bedding is associated with higher BT raw milk spore counts, suggesting that use of inorganic bedding materials may provide a potential option that producers could explore if raw milk with a low spore count is desired. Alternatively, if organic bedding materials are used, implementation of other management strategies to control spore counts in raw milk may be needed.

Used Bedding Spore Levels Are Directly Influenced by Unused Bedding Spore Levels and Indirectly Influenced by Bedding Materials

Our study identified that mesophilic and thermophilic spore levels in used bedding were directly positively influenced by spore levels in unused bedding. Although the direct effect of spore levels in unused bedding on used bedding spore levels is not surprising, these findings do support the importance of selecting unused bedding with low spore levels despite the likely addition of considerable spore loads to bedding during use (e.g., from deposition of fresh manure). Our data also indicate that some specific bedding materials indirectly influenced spore levels in used bedding through unused bedding spore levels. Specifically, organic bedding materials, including MNS and OO, indirectly influenced MSC and TSC levels in used bedding. In addition, ST indirectly influenced MSC levels in used bedding, whereas RS indirectly influenced TSC levels in used bedding; RS was the only inorganic bedding material that indirectly (through unused bedding spore levels) influenced any spore levels in used bedding. These findings are not surprising because our data also indicated that bedding material affected spore counts in unused bedding materials. These findings support the idea that spore levels in unused bedding influence those in used bedding and that this relationship is influenced by inherent properties of the bedding material. Combined with the predicted direct effect of specific organic bedding materials on spore levels in used bedding (as discussed in the previous section), this finding further supports the importance of organic bedding as a factor that increases the risk of high spore levels in dairy cow housing areas. This conclusion is consistent with other studies that identified organic bedding materials as having higher levels of mastitis-associated pathogens (Hogan et al., 1989; Godden et al., 2008).

Used Bedding Spore Levels Directly Affect BT Raw Milk Spore Levels

This study identified and quantified a direct relationship between spore levels in used bedding and raw milk: BT raw milk spore levels increase with used bedding

spore levels. Previous studies have investigated transmission of spores from environmental sources, including used bedding, to BT raw milk and have proposed that used bedding influences spore levels in raw milk via contamination of the udder and teats (Slaghuis et al., 1997; Magnusson et al., 2007a; Vissers et al., 2007a; Bava et al., 2017). In the case of TS farms where milking occurs in the housing area, spores in used bedding may also influence BT raw milk spore levels via contamination of the milking apparatus (e.g., in the event of a milking unit kick-off). Another proposed mechanism of bacterial transmission from used bedding to raw milk is via aerosolization of bedding during handling, distribution, or removal from the housing area, resulting in airborne dust particles carrying bacteria that may settle on feed or water that is ingested and thus may contaminate the udder and teats via manure (LeJeune and Kauffman, 2005). These airborne bedding dust particles carrying bacteria could also settle on milking equipment, depending on proximity to the milking area.

Reports on the relative contribution of bedding as a contamination source of spores in BT raw milk compared with other environmental sources (e.g., manure, soil, and silage) have been conflicting (McKinnon and Pettipher, 1983; Magnusson et al., 2007a; Vissers et al., 2007a,b). In addition to influencing bacterial levels in BT raw milk, the dairy farm environment has been predicted to drive individual raw milk and BT raw milk microbiota composition (Vacheyrou et al., 2011; Doyle et al., 2016; Metzger et al., 2018). In a pilot study evaluating milk sampling methods used in studies of the milk microbiota, Metzger et al. (2018) also identified differences in milk microbiome by bedding material (including NS, RS, SH, and MNS), suggesting that variation in milk microbiome may be influenced by the type of bedding material cows are exposed to. Using high-throughput DNA sequencing and a Bayesian inference algorithm, Doyle et al. (2016) identified used bedding, in addition to other environmental factors (i.e., teats, manure, and silage), as a contamination source of individual raw milk and BT raw milk for herds housed indoors. Given the number of studies supporting used bedding as an environmental source of spores in BT raw milk, future studies investigating dynamics of spore-forming bacteria on dairy farms should include used bedding as a potential source. However, data available to date already provide strong support for the idea that management of spore levels in used bedding materials represents a possible management strategy that could be tested, through intervention studies, on individual farms that need to achieve lower spore levels in their raw milk.

Bedding and Farm Management Practices Are Associated with Spore Levels in Bedding and BT Raw Milk

In addition to the effect of bedding type on spore levels in unused and used bedding as well as BT raw milk, we identified several bedding and farm management practices that were positively or negatively associated with spore levels in unused bedding, used bedding, and BT raw milk. Surprisingly, the only management practice found to be associated with spore levels in unused bedding was frequency of adding unused bedding to stalls. Specifically, more frequent addition of unused bedding to stalls was associated with lower MSC (for TS only) and TSC (for TS and DL) in unused bedding. Although it is not plausible that the practice of more frequently adding unused bedding to a stall will decrease spore levels in the unused bedding, this finding could be explained if more frequent bedding addition is associated with shorter storage of bedding on the farm (as bedding is used up more quickly); the farm management survey did not allow us to reliably assess storage time of bedding on the farm directly. Shorter storage of unused bedding may lead to unused bedding with lower spore counts, particularly if longer on-farm storage either facilitates growth of sporeformers (and sporulation) or leads to an increased risk of contamination from external sources.

With regard to used bedding, we identified 3 practices as being associated with decreased TSC levels: (1) frequency of adding unused bedding to stalls, (2) frequency of raking or tilling surfaces in DL or BP housing, and (3) housing in DL. These practices only affected TSC counts in farms with BP and DL housing compared with farms with FS housing. Findings 1 and 2 are consistent with previous studies that reported that maintaining housing cleanliness is important for minimizing bedding bacterial levels (Magnusson et al., 2007b; Sorter et al., 2014; Rowbotham and Ruegg, 2016). Our observation that housing in DL was associated with lower used bedding TSC compared with FS housing was surprising but could be attributed to DL-associated bedding and farm management factors that were not included in our models or could be due to other characteristics that are unique to or associated with DL. We also identified 1 management practice (frequency of removing manure from the housing area) as being associated with higher TSC in used bedding; this practice only affected TSC in farms with DL housing. This finding may seem somewhat counterintuitive, particularly because a previous study conducted at an FS farm using sawdust for bedding found that remov-

ing used bedding material from stalls and replacing it with unused bedding initially reduced *B. cereus* spore levels in bedding in the stalls, although the same spore levels as before replacement were reached after 4 to 6 wk (Magnusson et al., 2007b). However, several studies have suggested that bacteria in manure may be transferred via aerosolization (Ravva et al., 2011; Berry et al., 2015). As DL farms are typically located in hot, dry regions (Tresoldi et al., 2016), removing manure from the yard may generate dust, potentially resulting in enhanced transmission of spores when manure is removed from housing areas. Overall, our findings suggest that bedding management strategies can affect spore levels in used bedding, which is important because spore counts in used bedding are associated with spore counts in BT raw milk. Although the data reported here suggest that more frequent addition of unused bedding as well as more frequent raking or tilling of surfaces in DL or BP housing may reduce used bedding spore counts, follow-up studies that specifically assess the effect of changes in use of bedding management practices on bedding and BT raw milk spore counts will be important.

Finally, we also identified 2 management practices that were directly associated with increased BT raw milk spore levels: (1) a higher proportion of cows in the housing area with a hygiene score of 3 (i.e., 10–30% of udder and hind leg surface area was covered with dirt) was associated with higher MSC and TSC in BT raw milk (compared with a hygiene score of 1), and (2) housing in BP (compared with housing in FS) was associated with higher MSC in BT raw milk. Although further studies will be needed to confirm finding 1, several previous studies have reported a positive association between cow hygiene scores in the milking area and spore levels in BT raw milk (Masiello et al., 2014; Miller et al., 2015a; Zucali et al., 2015). As such, producers interested in low-spore raw milk should focus on maintaining cow cleanliness not only in the milking area but also in the housing area. Additionally, follow-up studies assessing potential associations between hygiene scores in the housing area, hygiene scores in the milking area, and BT raw milk spore levels may inform strategies for maintaining cow cleanliness, thereby reducing BT raw milk spore levels. Regarding finding 2, previous studies have shown that lying times for lactating cows housed in BP are longer than FS, with differences ranging from 33 min (Fregonesi et al., 2009) to 3 h (Phillips and Schofield, 1994). As such, longer lying times on farms with BP housing may increase the likelihood for attachment of spores from used bedding to the udder and potential subsequent transfer to the BT raw milk. We also identified 2 practices that were indirectly

and directly associated with decreased BT raw milk TSC. Specifically, (1) more frequent addition of unused bedding to pens was indirectly associated with slightly lower BT raw milk TSC through used bedding TSC (for BP only), and (2) removing udder hair by clipping or singeing at least once per lactation was directly associated with lower BT raw milk TSC compared with not removing udder hair. Finding 1 further supports the idea that more frequent addition of unused bedding may reduce BT raw milk spore counts, in particular for producers housing in BP. Finding 2 is consistent with a previous report from a study conducted at dairy farms across Prince Edward Island that identified udder clipping as being associated with lower BT raw milk total aerobic counts (Elmoslemany et al., 2009) and coliform counts (Elmoslemany et al., 2010). Although the specific method for udder hair removal (i.e., clipping or singeing) was not included in our models, 2 studies conducted at Washington State University's Dairy Center reported that udder singeing did not have a significant effect on BT raw milk bacterial counts (Silk et al., 2003; Fox, 2016); hence, it is possible that associations between udder hair removal and BT raw milk bacterial levels may differ according to method used for removing udder hair. Furthermore, the effect of removing udder hair (by clipping, singeing, or other practices for udder hair removal) may differ for vegetative bacterial cells versus spores. For farms regulated by the US grade A Pasteurized Milk Ordinance, udder clipping is mandatory to facilitate udder cleaning (FDA, 2013). Thus, our observation that udder hair removal by clipping or singeing was associated with lower BT raw milk TSC may either be explained by an actual measured effect of udder hair removal on bacterial levels or instead serve as a proxy for adherence to milking-specific regulations in the Pasteurized Milk Ordinance.

CONCLUSIONS

Overall, this study supports the idea that maintaining low spore levels in unused and used bedding materials may be important for producing low-spore raw milk. Our study also identified housing cleanliness, including frequent addition of unused bedding to the housing area, as being important for maintaining low spore levels in both unused and used bedding. Notably, organic bedding materials (compared with inorganic bedding materials) are associated with higher spore levels in unused and used beddings and may be indirectly associated with higher BT raw milk spore levels. Given this finding, future research should focus on identifying practices specifically aimed at maintaining low spore levels in organic bedding materials. Our

study also identified that mesophilic and thermophilic spore levels in BT raw milk are influenced by used bedding spore levels, which are in turn influenced by unused bedding spore levels. Based on these results, producers who require low-spore raw milk (e.g., to receive premium payments) may consider monitoring spore levels (e.g., test incoming bedding shipments) and implementing practices to reduce bedding spore load. The mesophilic and thermophilic spore levels in unused bedding, used bedding, and BT raw milk reported here may serve as a baseline for producers to evaluate their own bedding spore levels, but additional studies are needed to improve our understanding of the mechanisms driving the relative effects of the identified bedding management practices. Importantly, our study identified associations, which may or may not represent cause-and-effect relationships; hence, our study results should not be seen as providing recommendations for specific interventions or control strategies that should be implemented. Rather, our findings identify potential interventions and control strategies that can and need to be further tested, including on farms that may have a need to achieve lower spore levels in raw milk.

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