J. Dairy Sci. 99:6594–6608
http://dx.doi.org/10.3168/jds.2015-10674

Bacterial counts on teat skin and in new sand, recycled sand, and recycled manure solids used as bedding in freestalls

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ABSTRACT

On modern dairy farms, environmental mastitis pathogens are usually the predominant cause of mastitis, and bedding often serves as a point of exposure to these organisms. The objective of this longitudinal study was to determine bacterial populations of 4 different bedding types [deep-bedded new sand (NES), deep-bedded recycled sand (RS), deep-bedded manure solids (DBMS), and shallow-bedded manure solids over foam core mattresses (SBMS)] and of teat skin swabs of primarily primiparous cows housed in a single facility over all 4 seasons. Samples of bedding were collected weekly (n = 49 wk) from pens that each contained 32 lactating dairy cows. Throughout the length of the same period, composite swabs of teat skin were collected weekly from all cows before and after premilking teat sanitation. Median numbers of streptococci and streptococci-like organisms (SSLO) were $>8.6 \times 10^6$ cfu/g and $>6.9 \times 10^3$ cfu/teat swab for all bedding types and teat swabs, respectively. Numbers of SSLO were greatest in samples of SBMS (2.1×10^8 cfu/g) and least in samples of NES (8.6×10^6 cfu/g), RS (1.3×10^7 cfu/g), and DBMS (1.7×10^7 cfu/g). Numbers of gram-negative bacteria in bedding (5.5×10^4 to 1.2×10^7 cfu/g) were fewer than numbers of SSLO (8.6×10^6 to 2.1×10^8 cfu/g). Numbers of coliform bacteria were greatest in samples of DBMS (2.2×10^6 cfu/g) and least in samples of NES (3.6×10^3 cfu/g). In general, the relative number of bacteria on teat skin corresponded to exposure in bedding. Numbers of gram-negative bacteria recovered from prepreparation teat swabs were greatest for cows bedded with DBMS (1.0×10^4 cfu/swab) and RS (2.5×10^3 cfu/swab) and least for cows bedded with NES (5.8×10^2 cfu/swab). Median numbers of coliform and *Klebsiella* spp. recovered from prepreparation teat swabs were below the limit of detection for all cows except those bedded with DBMS. Numbers of SSLO recovered from prepreparation teat swabs were least for cows bedded with DBMS (6.9×10^3 cfu/swab) and greatest for cows bedded with RS (5.1×10^4 cfu/swab) or SBMS (1.6×10^5 cfu/swab). The numbers of all types of measured bacteria (total gram-negative, coliforms, *Klebsiella* spp., SSLO) on postpreparation teat swabs were reduced by up to 2.6 logs from numbers of bacteria on prepreparation swabs, verifying effective preparation procedures. Significant correlations between bacterial counts of bedding samples and teat skin swabs were observed for several types of bacteria. As compared with other bedding types, the least amount of gram-negative bacteria were recovered from NES and may indicate that cows on NES have a reduced risk of exposure to pathogens that are typically a cause of clinical mastitis. In contrast, exposure to large numbers of SSLO was consistent across all bedding types and may indicate that risk of subclinical mastitis typically associated with streptococci is not as influenced by bedding type; however, significantly greater numbers of SSLO were found in SBMS than in other bedding types. These findings indicate that use of different bedding types results in exposure to different distributions of mastitis pathogens that may alter the proportion of etiologies of clinical mastitis, although the incidence rate of clinical mastitis did not differ among bedding types.

Key words: mastitis, dairy cow, bacteria, milk quality

INTRODUCTION

For decades, mastitis has been considered to be the most economically important and most commonly occurring disease of dairy cattle (Blackburn, 1958; Janzen, 1970; Hogeveen et al., 2011), and it continues to contribute to decreased profits for dairy farms throughout the world. Mastitis pathogens are frequently categorized as environmental or contagious based upon their primary reservoir and point of exposure (Smith and Hogan, 2001). Exposure to contagious mastitis often occurs during milking when teats of healthy cows are exposed to bacteria in milk that originated

Received November 23, 2015.
Accepted April 22, 2016.
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from infected quarters. The prevalence of contagious IMI has decreased due to adoption of recommended milking practices and selective culling of chronically infected cows. Exposure to environmental pathogens occurs when teats are exposed to large numbers of opportunistic organisms found in the animals' housing areas. As contagious pathogens have been controlled, environmental pathogens have come to account for the majority of IMI (Makovec and Ruegg, 2003). Possible sources of exposure include bacteria found in bedding, manure, and mud (Schreiner and Ruegg, 2003; DeVries et al., 2012; Hogan and Smith, 2012). Reducing bacterial exposure at the teat end is an important aspect of prevention of environmental mastitis. Dairy cattle spend 40 to 65% of their time lying down, and during these periods their teats may come in direct contact with bacteria found in bedding (Tucker and Weary, 2004; Cook et al., 2005; Hogan and Smith, 2012). The incidence of clinical mastitis has been shown to be associated with bacterial populations on teat ends (Neave et al., 1966), and teat-end bacterial populations have been correlated with bacterial populations found in bedding (Rendos et al., 1975; Hogan et al., 1999; Zdanowicz et al., 2004). Thus, to control environmental mastitis, dairy producers often focus on reducing exposure to pathogens found in bedding (Hillerton and Berry, 2003; Hogan and Smith, 2012).

Herds containing ≥200 milk cows currently produce 75% of all milk in the United States, and those containing ≥500 cows produce 63% of US milk (USDA-NASS, 2014). Bedding used on larger Wisconsin (**WI**) dairy farms includes fresh (58%) or recycled (10%) sand, organic materials (primarily wood products on top of mattresses; 22%), and manure products (10%; Rowbotham and Ruegg, 2015). Differences in the quality and quantity of milk produced per cow on larger WI dairy farms have been associated with the type of bedding used, and potential economic advantages exist for using inorganic bedding (Rowbotham and Ruegg, 2015). Whereas potential cost savings may be had for herds that use recycled bedding materials, those savings must be evaluated relative to potential differences in exposure to mastitis pathogens. The effect of different types of bedding on bacterial populations on teat skin is not well defined and is especially relevant for dairy farmers who must select bedding based on both cow management issues and environmental restrictions on manure management.

Numbers of streptococci on teats have been reported to be several log units greater than numbers of gram-negative or coliform bacteria (Rendos et al., 1975; Hogan et al., 1990). Correlations between bedding and teat-end bacterial populations differ between sand and sawdust (Zdanowicz et al., 2004), which emphasizes the importance of studying both bedding and teat skin bacterial populations when comparing different bedding materials. Teat-end bacterial populations have also been associated with bedding DM (Proietto, et al., 2013). Most studies of bacterial populations in bedding have been of short duration (3 to 9 wk; Fairchild et al., 1982; Kristula et al., 2008; Sorter et al., 2014), with several focusing on bedding additives or treatments (Hogan et al., 1999, 2007, 2012). Few studies have compared bacterial populations in fresh and recycled sand (Kristula et al., 2005). In an observational study conducted on commercial dairy farms using clean or recycled sand (Kristula et al., 2005), seasonal differences in bacterial growth patterns were observed between summer and winter, but the study did not include organic bedding nor quantify teat skin bacteria. The objective of the current longitudinal study was to determine bacterial populations of 4 different bedding types [deep-bedded new sand (**NES**), deep-bedded recycled sand (**RS**), deep-bedded manure solids (**DBMS**), and shallow-bedded manure solids over foam core mattresses (**SBMS**)] and on teat skin swabs of primarily primiparous cows housed in a single facility over all 4 seasons.

MATERIALS AND METHODS

Experimental Design and Quarter Enrollment Criteria

The experiment was conducted at the University of Wisconsin–Madison, Marshfield Agricultural Research Station from January to December, 2013. Four bedding types were tested in a freestall barn 29.3 m wide by 59.4 m long with 4.3-m tall open side walls that contained identical pens (n = 4), each 11.3 m wide by 26.1 m long. Each pen housed up to 32 lactating cows in 2 rows of 16 head-to-head freestalls. Freestall dimensions were: 1.65 m from rear curb to brisket locator, 1.78 m from rear curb to neck rail, 0.23 m curb height, and 1.28 m width (divider mounting on center). Alleys between freestalls and feed bunks were 4.04 m wide and alleys between freestalls and outside walls were 2.44 m wide. Throughout the period of the trial, each of the 4 pens contained a single type of bedding material: (1) NES, which was deep bedded, previously unused pit sand; (2) RS, which was deep bedded sand recycled on the farm using a screw-type sand separator designed to recover 80 to 90% of sand from manure for reuse as bedding (McLanahan, Hollidaysburg, PA); (3) DBMS, which was recycled on the farm using a screen press (PSS 1.2–520 FAN Separator, Bauer Group, Marktschorgast, Germany); and (4) SBMS, which was the same recycled manure solids as DBMS, shallow bedded over foam-core mattresses. Twice daily, as cows were milked, bedding was manually groomed and alleys were scraped. Fresh

bedding was added to all stalls on Tuesday and Friday afternoons. All cows were fed the same TMR consisting of 20.8% corn silage, 17.5% alfalfa haylage, 5.8% alfalfa hay, 21.4% high-moisture corn, 20% soybean protein mix, 5.2% corn gluten pellets, 3.6% cottonseed, and 5.8% liquid sugar and mineral mix.

Sample Collection and Bacterial Analysis of Samples

Used Bedding. Beginning on January 25 and continuing until December 27, 2013, researchers collected bedding samples each Friday before new bedding was added to the stalls. Each week, 16 subsamples were collected from each pen and combined to form a single composite bedding sample for each pen. Subsamples were composed of bedding material taken from the top 8 cm of bedding from 4 locations in the back one-third of each of 4 stalls per pen. The 4 stalls varied each week and were preselected using a table of random numbers. The 4 sampling locations per stall also varied. To select the locations, a researcher centered a metal grid containing 42 uniformly sized locations (6 rows each 14.5 cm tall by 7 columns each 19.5 cm wide) in the back of each assigned stall and used a gloved hand to collect bedding from each of 4 randomly assigned locations. After the samples were combined, they were immediately frozen (for 1–4 wk in a non-frost-free freezer at −25°C), then transported to the University of Wisconsin Milk Quality Laboratory for testing.

Bedding samples were allowed to thaw at room temperature. Each bedding sample of approximately 0.25 L was thoroughly mixed in the 0.95-L sample bag after thawing. To prepare an initial 1:10 dilution, 10 g of each bedding sample were suspended in 90 mL of sterile PBS, manually agitated for 60 s, and allowed to settle for 15 to 20 min to allow pipetting of the liquid rinse after bedding particles settled or floated. Bacterial colonies were enumerated using previously described methods that involved plating serial dilutions onto selective agar media (Hogan et al., 2007). Briefly, duplicate serial dilutions of the liquid were prepared and four 10-μL inoculations of each duplicate dilution ($1:10^1$ to $1:10^5$) were plated on the surface of MacConkey agar (BD, Sparks, MD), MacConkey-inositol-carbenicillin agar (MacConkey agar base supplemented with 5 g of myo-inositol/20 g of MacConkey, and 75 μg/mL of carbenicillin as per Hogan et al., 2007), and modified Edwards agar (Oxoid, Basingstoke, UK) supplemented with colistin sulfate and 5% plasma and oxolinic acid as per Hogan et al. (2007). Plates were incubated for 24 h at 37°C. Colony-forming units were enumerated as gram-negative (all colonies on MacConkey agar), coliforms (lactose-positive colonies on MacConkey agar), *Klebsiella* spp. (pink and red colonies on MacConkey-inositol-carbenicillin agar), and streptococci and streptococci-like organisms (**SSLO**; all colonies on modified Edwards agar; Sawant et al., 2002). For each bacterial type, the plate with the least dilution that contained countable (≤50 cfu/inoculation) colony-forming units was enumerated. The average number of colonies from each duplicate dilution was calculated as the mean number of colonies on the four 10-μL inoculations. The final number of colonies was the mean of the average number of colonies from the 2 duplicate dilutions. The numbers of colonies were log-transformed and analyzed as $\log_{10}$ (cfu + 1) per gram of bedding. After thorough mixing and removal of sample for bacteriological examination, bedding from samples was dumped into clean pans (small bread loaf pans, approximately 0.5 L in volume) for placement into DM oven. Samples of NES and RS had considerably more mass than manure bedding samples. To determine DM content, 75 to 500 g of each bedding sample were placed in a convection oven for 48 h at 55°C.

Unused Bedding. Unused bedding was sampled once monthly for 10 of 12 mo of 2013 by comingling 5 random grab samples from piles of unused bedding. Unused bedding samples were handled and tested using the same methods as used bedding samples.

Teat Swabs. During the afternoon milking on each Friday that bedding samples were collected, composite teat swabs were collected before and after premilking sanitization from all cows in each pen. Composite teat swab samples were accumulated by researchers who swabbed one teat of each cow (n = 32 teats per pen) using a single sterile 12-ply 4 × 4 cotton gauze pad moistened with PBS per teat and alternating teats between consecutive cows. Swabs were passed down the side of the teat from base to apex, then 3 times across the teat apex before depositing in a sterile specimen cup. Two composite premilking samples (**PRSWAB**), each consisting of 16 individual teat swabs per pen, were collected. Two composite samples were collected per pen to minimize the possibility of contamination on farm because the milking parlor held 16 cows and there were 32 cows in each pen. Milking technicians then performed routine premilking preparation that included application of a 0.5% iodine based predip sanitizer using a dip cup, removal and examination of foremilk, and drying teats using an individual cloth towel per cow. After premilking sanitization, a composite teat swab sample (**POSWAB**) per pen was collected using the same methods as described for the PRSWAB. Composite teat swabs samples (n = 2 PRSWAB and 2 POSWAB) were frozen for up to 4 wk in a non-frost-free freezer at −25°C before processing at the University of Wisconsin Milk Quality Laboratory. All teat swab samples were collected by the same 2 researchers.

Composite teat swab samples were allowed to thaw at room temperature. For both PRSWAB and POSWAB, a single composite teat swab for each pen was prepared by combining each pair of collected composite samples and suspending in sterile PBS in 1-L sterile plastic bags and stomaching for 60 s. Serial dilutions of the rinse solutions from the suspensions were plated and bacterial colonies were enumerated using the same methods as described for the bedding samples. The numbers of colonies were expressed as $\log_{10}$ colony-forming units per teat swab. For each sample where PRSWAB was not below the limit of detection, the log reduction (**RED**) in the number of teat skin swab bacteria was calculated as RED = PRSWAB – POSWAB.

Statistical Analysis

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC). Pen was the experimental unit used for analysis. Descriptive statistics were examined using PROC UNIVARIATE and PROC SGPLOT. For all multivariate models, model fit was assessed by the Akaike information criterion (Akaike, 1969) and multiple comparisons were adjusted using Tukey-Kramer. All means are presented as mean ± standard error.

To test the null hypothesis that no differences existed in bacterial populations in bedding or teats swabs based on bedding type, multivariate Tobit regression models were constructed using PROC LIFEREG. Tobit regression is a combination of a censored regression with all the threshold observations truncated and a univariate probit model with the one-zero distinction at the threshold point (Greene, 2003). The equation for each model was

$$\log_{10}BC_{ijk} = \alpha + \text{bedding}_i + \text{season}_j + \text{bedding} \times \text{season}_{ij} + \varepsilon_{ijk},$$

where $\log10BC_{ijk}$ was the base 10 logarithm of the bacterial colony-forming unit of interest + 1 (total gram-negative bacteria, coliform, *Klebsiella* spp., SSLO) in bedding or recovered from teat swabs; α was the intercept; bedding_i was NS, RS, DBMS, or SBMS; season_j was season of sampling (spring, summer, fall, winter); bedding × season_{ij} was used as an interaction term; and ε_{ijk} was residual error. Seasons were defined as spring (March to May), summer (June to August), fall (September to November), and winter (December to February).

To test the null hypothesis that there was no difference in RED among bedding types, 4 separate linear models (one for each bacterial type) were constructed using PROC MIXED. In these models, RED was the response variable and bedding type, season, and the interaction of bedding and season were used as explanatory variables. Only RED with PRSWAB >0 were offered to the models.

To test the null hypothesis that there were no differences in DM among bedding types and seasons, PROC MIXED was used to perform multiple regression between DM (response variable) and bedding type, season, and the interaction of bedding type and season (explanatory variables). To test the null hypothesis that there was no difference in DM within bedding types among seasons, PROC MIXED was used to perform 4 single linear regression models, one for each bedding type, with DM as the response variable and season as the explanatory variable.

To test the null hypothesis that average DHI test day DIM, SCC, and kilograms of milk produced daily did not differ in cows housed in pens with different bedding types, 3 separate repeated measures regression models with autoregressive covariance structures were constructed using PROC MIXED. The explanatory variable in each model was bedding type and the response variables were $(\text{DIM})^{1/2}$, SCS, or kilograms of milk. Spearman ranked correlations between bacterial populations in bedding and on teat skin were analyzed for each type of bacteria (gram-negative, coliform, *Klebsiella* spp., SSLO) among results pooled for all bedding types and separately for each bedding (NS, RS, DBMS, SBMS) using PROC CORR. All procedures were approved by the Animal Care and Use Committee for the College of Agricultural and Life Sciences of the University of Wisconsin–Madison.

RESULTS

Description of Herd

This University of Wisconsin research facility was initially populated with multiparous cows from other University of Wisconsin facilities that had been randomly assigned to the 4 treatment pens in 2012. Prior to our experiment, as these cows reached the end of lactation, they were replaced with primiparous Holsteins randomly assigned to enter the pens after they calved. At the beginning of our experiment, the pens were populated with primiparous Holsteins (n = 96), multiparous cows (n = 15), and 1 primiparous Jersey (Table 1). During the experiment cows (n = 136) entered the pens to maintain populations as cows reached the end of their lactation or were culled. Cows entering during the experiment were primarily primiparous Holsteins (n = 114; Table 1) and during the experiment, 85% of the cows in the pens were primiparous Holsteins (n =

210). Cows were evenly distributed among pens and the number of cows per pen never exceeded 33 (Table 1). Least squares means DHI test-day milk production did not vary based on bedding type and were 31.3 ± 0.9, 31.2 ± 0.9, 31.7 ± 1.0, and 30.5 ± 0.9 kg per cow per day for cows bedded with NES, RS, DBMS, and SBMS, respectively ($P = 0.791$). Least squares means DHI test-day SCS did not vary based on bedding type and were 2.2 ± 0.15, 2.4 ± 0.15, 1.9 ± 0.15, and 2.2 ± 0.15 for cows bedded with NES, RS, DBMS, and SBMS, respectively ($P = 0.083$). When adjusted for multiple comparisons, SCS of cows bedded with DBMS tended to be less than SCS of cows bedded with RS ($P = 0.054$), with no tendencies for differences among other bedding types ($P \geq 0.344$). Least squares means DHI test-day DIM did not vary based on bedding type and were 160 ± 20, 187 ± 20, 115 ± 18, and 150 ± 20 d for cows bedded with NES, RS, DBMS, and SBMS, respectively ($P = 0.060$). When adjusted for multiple comparisons, DIM of cows bedded with DBMS was lesser than DIM of cows bedded with RS ($P = 0.038$), with no differences among other bedding types ($P \geq 0.275$). Associations among bedding type and subclinical and clinical mastitis observed during this same time period are reported in a companion paper (Rowbotham and Ruegg, 2016).

Bacterial Populations in Bedding and on Teat Skin

Used Bedding. Weekly composite bedding samples (n = 49) were analyzed for each bedding type. Numbers of gram-negative bacteria in bedding were above the limit of detection (125 cfu/g) in almost all bedding samples (Table 2). Likewise, the numbers of coliform bacteria (a subset of gram-negative bacteria) were above the limit of detection in all but 2, 1, and 4 samples of NES, RS, and SBMS, respectively. Numbers of *Klebsiella* spp., (a type of coliform bacteria) were below the detection limit detection for 25% of samples of NES and SBMS, but were identified in all but 2 samples (4%) of DBMS. Numbers of SSLO in bedding were above the limit of detection for all samples of all bedding types (Table 2).

The median number of total gram-negative bacteria in DBMS (1.2×10^7 cfu/g) was 13 times as great as the median number in SBMS and 60 and 216 times as great as the median numbers in RS and NES, respectively (Table 3). Except for equal numbers of *Klebsiella* spp. in NES and SBMS, quantities of gram-negative, coliform, and *Klebsiella* spp. bacteria were least in NES, intermediate in RS and SBMS, and greatest in DBMS ($P < 0.001$). With the exception of DBMS, relatively few coliform (including the subset of *Klebsiella* spp.) were recovered from bedding. Mean numbers of coliform represented a small proportion [7, 6, 6, and 2%; sample calculation: $10^{3.57 - 4.73} \times 100 = 6.9\%$ of gram-negative bacteria in new sand (4.73 $\log_{10}$ cfu/g) are coliform (3.57 $\log_{10}$ cfu/g] of total gram-negative bacteria in NES, RS, DBMS, and SBMS, respectively, indicating that other types of gram-negative bacteria contributed the majority of gram-negative organisms recovered from all types of bedding. Numbers of total gram-negative bacteria, coliforms, and *Klebsiella* spp. were significantly greater in the summer than in other seasons ($P < 0.001$; Table 4) for all bedding types. The interaction between bedding type and season did not remain in any of the bedding bacteria count models.

The median number of SSLO was $>8.6 \times 10^6$ cfu/g in all bedding types and exceeded 10^8 cfu/g for SBMS (Table 3). Least squares means numbers of SSLO from the regression model were greatest in SBMS, which contained >9 times [the relationship was calculated as $10^{8.17 - 7.21} = 9.12$ times as many streptococci in shallow-bedded manure solids (8.17 $\log_{10}$ cfu/g) as in recycled sand (7.21 $\log_{10}$ cfu/g)] as many colony-forming units per gram as any other bedding. Bedding samples contained >7 $\log_{10}$ cfu/g of SSLO in all seasons and the number of SSLO did not vary seasonally ($P =$

Table 1. Herd population (no. of cows) in a trial of associations among bacterial populations in bedding and on teat skin in a controlled experiment in a freestall barn in Wisconsin

Item	New sand	Recycled sand	Deep-bedded manure solids	Shallow-bedded manure solids[1]	Total
Initial pen populations (Jan. 1, 2013)	33	30	17	32	112
Holstein, lactation = 1	28	25	12	31	96
Holstein, lactation ≥2	4	4	4	—[2]	12
Other breeds	1			1	4
Cows entering pens after Jan. 1, 2013	37	31	40	28	136
Holstein, lactation = 1	29	28	31	26	114
Holstein, lactation ≥2	7	3	7	—	17
Other breeds	1	—	2	2	5

[1]Over foam core mattresses.

[2]No cows in this category.

Table 2. Bacterial counts below the limit of detection[1] from weekly evaluation of bedding (n = 49) and teat skin swabs (n = 49) of cows in a controlled experiment in a freestall barn in Wisconsin

Bacterial group, % of samples	Sample source	New sand	Recycled sand	Deep-bedded manure solids	Shallow-bedded manure solids[2]
Gram-negative	Bedding	2.0	2.0	0.0	0.0
	PRSWAB[3]	20.4	4.1	4.1	4.1
	POSWAB[4]	55.1	38.8	28.6	38.8
Coliform	Bedding	4.1	2.0	0.0	8.2
	PRSWAB	79.6	61.2	30.6	75.5
	POSWAB	91.8	87.8	91.8	87.8
Klebsiella, spp.	Bedding	24.5	16.3	4.1	24.5
	PRSWAB	95.9	73.5	34.7	93.9
	POSWAB	95.9	98.0	93.9	93.9
SSLO[5]	Bedding	0.0	0.0	0.0	0.0
	PRSWAB	4.1	0.0	6.1	4.1
	POSWAB	18.4	8.2	34.7	4.1

[1]Limit of detection 125 cfu/g for bedding and 77 cfu/teat swab.

[2]Over foam-core mattresses.

[3]Before premilking sanitization teat swab.

[4]After premilking sanitization teat swab.

[5]Streptococci and streptococci-like organisms isolated on modified Edwards medium (Oxoid, Basingstoke, UK; supplemented with colistin sulfate and 5% plasma and oxolinic acid as per Hogan et al., 2007).

0.702; Table 4). For NES, RS, and SBMS the numbers of SSLO were 2.0 to 2.4 $\log_{10}$ cfu/g greater than the numbers of total gram-negative bacteria ($P < 0.001$), whereas numbers of SSLO and total gram-negative bacteria were similar in DBMS ($P = 0.056$; Table 3).

The magnitude of the differences in the number of bacteria also varied among bedding types and bacterial groups (Table 3). There was a >2 $\log_{10}$ cfu/g difference in the number of total gram-negative bacteria, coliforms, or *Klebsiella* spp. between NES and DBMS. In contrast, there was only a 0.47 to 0.72 $\log_{10}$ cfu/g difference in the number of gram-negative bacteria, coliforms, or *Klebsiella* spp. between NES and RS and 1.02 to 1.74 $\log_{10}$ cfu/g difference between RS and DBMS, indicating that even though the number of these bacteria varied significantly among bedding types, the

Table 3. Number of gram-negative bacteria, coliforms, *Klebsiella* spp., and SSLO[1] in 4 different bedding types and recovered from premilking teat swabs, stratified by bedding type, from weekly (n = 49) sampling in a controlled experiment in a freestall barn in Wisconsin

Bacterial group	Sample source	New sand	Recycled sand	Deep-bedded manure solids	Shallow-bedded manure solids[2]
Raw values, median					
Gram-negative	Bedding[3]	55,000	197,000	11,875,000	912,500
	PRSWAB[3]	575	2,454	10,315	2,837
Coliform	Bedding	3,625	15,000	2,250,000	26,250
	PRSWAB	0	0	345	0
Klebsiella spp.	Bedding	375	2,625	525,000	1,375
	PRSWAB	0	0	153	0
SSLO	Bedding	8,625,000	13,250,000	16,875,000	206,250,000
	PRSWAB	19,762	51,226	6,910	159,619
LSM[4] (SE)					
Gram-negative	Bedding[5]	4.73^{d} (0.122)	5.26^{c} (0.122)	6.80^{a} (0.122)	5.78^{b} (0.122)
	PRSWAB[5]	2.69^{e} (0.119)	3.57^{ab} (0.116)	3.97^{a} (0.116)	3.34^{b} (0.116)
Coliform	Bedding	3.57^{c} (0.135)	4.04^{b} (0.135)	5.61^{a} (0.135)	4.13^{b} (0.135)
	PRSWAB	1.11^{b} (0.171)	1.54^{b} (0.141)	2.36^{a} (0.118)	1.31^{b} (0.141)
Klebsiella spp.	Bedding	2.55^{c} (0.176)	3.27^{b} (0.171)	5.01^{a} (0.165)	2.94^{bc} (0.172)
	PRSWAB	0.44^{c} (0.292)	1.35^{b} (0.157)	2.08^{a} (0.114)	0.69^{c} (0.243)
SSLO	Bedding	6.88^{c} (0.078)	7.21^{b} (0.078)	7.08^{bc} (0.078)	8.17^{a} (0.078)
	PRSWAB	4.28^{b} (0.087)	4.82^{a} (0.087)	3.82^{c} (0.087)	5.05^{a} (0.087)

[1]Streptococci and streptococci-like organisms isolated on modified Edwards medium (Oxoid, Basingstoke, UK; supplemented with colistin sulfate and 5% plasma and oxolinic acid as per Hogan et al., 2007).

[2]Over foam-core mattresses.

[3]Bedding values in colony-forming units per gram; PRSWAB = teat swab values (cfu/swab) collected before premilking preparation.

[4]From regression models.

[5]Bedding values in Log_{10}(cfu/g + 1); PRSWAB in Log_{10}(cfu/swab + 1).

Table 4. Number of gram-negative bacteria, coliforms, *Klebsiella* spp., and SSLO[1] in 4 different bedding types and recovered from premilking teat swabs, stratified by season,[2] from weekly (n = 49) sampling in a controlled experiment in a freestall barn in Wisconsin

Bacterial group	Sample source	Spring	Summer	Fall	Winter
Raw values, median					
Gram-negative	Bedding[3]	426,250	925,000	356,250	73,229
	PRSWAB[3]	5,176	1,457	3,183	19,402
Coliform	Bedding	6,625	237,500	25,000	2,313
	PRSWAB	0	77	0	0
Klebsiella spp.	Bedding	1,313	32,500	2,125	125
	PRSWAB	0	0	0	0
SSLO	Bedding	16,375,000	13,000,000	24,062,500	12,500,000
	PRSWAB	46,711	20,592	25,981	340,521
LSM[4] (SE)					
Gram-negative	Bedding[5]	5.59^{xy} (0.114)	6.02^{w} (0.118)	5.70^{x} (0.118)	5.25^{y} (0.142)
	PRSWAB[5]	3.59^{w} (0.108)	3.13^{x} (0.113)	3.17^{x} (0.113)	3.67^{w} (0.137)
Coliform	Bedding	4.09^{y} (0.125)	5.41^{w} (0.130)	4.64^{x} (0.130)	3.22^{z} (0.158)
	PRSWAB	1.00^{x} (0.176)	1.89^{w} (0.123)	1.82^{w} (0.129)	1.62^{w} (0.161)
Klebsiella spp.	Bedding	3.19^{y} (0.158)	4.70^{w} (0.159)	3.79^{x} (0.161)	2.09^{z} (0.215)
	PRSWAB	0.73^{x} (0.226)	1.49^{w} (0.153)	1.14^{wx} (0.186)	1.20^{wx} (0.193)
SSLO	Bedding	7.34 (0.073)	7.37 (0.076)	7.26 (0.076)	7.38 (0.091)
	PRSWAB	4.68^{w} (0.081)	4.17^{x} (0.084)	4.23^{x} (0.084)	4.87^{w} (0.101)

[a–d]Means within a row with different superscripts differ among bedding types (Tukey adjusted $P < 0.05$).

[w–z]Means within a row with different superscripts differ among seasons (Tukey adjusted $P < 0.05$).

[1]Streptococci and streptococci-like organisms isolated on modified Edwards medium (Oxoid, Basingstoke, UK; supplemented with colistin sulfate and 5% plasma and oxolinic acid as per Hogan et al., 2007).

[2]Spring (March to May), summer (June to August), fall (September to October), winter (November to January).

[3]Bedding values in colony-forming units per gram; PRSWAB = teat swab values (cfu/swab) collected before premilking preparation.

[4]From regression models.

[5]Bedding values in Log_{10}(cfu/g + 1); PRSWAB in Log_{10}(cfu/swab + 1).

quantity of bacteria in RS was more similar to NES than to DBMS. In contrast, the difference in the quantity of SSLO among NES, RS, and DBMS was only 0.33 log_{10} cfu/g. This lesser difference in numbers of SSLO among bedding types as compared with numbers of gram-negative bacteria among bedding types is due to large numbers of SSLO found in all bedding types, whereas only DBMS had a mean $>9.2 \times 10^5$ cfu/g of total gram-negative bacteria. Overall, the magnitude of the differences in numbers of gram-negative bacteria among seasons was smaller (<0.8 log_{10} cfu/g; Table 4) than among bedding types. Two exceptions were the difference between summer and winter in numbers of coliform and *Klebsiella* spp., which were >2 log_{10} cfu/g, similar to the differences in the magnitude of these same populations between NES and DBMS.

When associations of DM were compared among bedding types, seasons, and bedding type by season interaction in a single model, the interaction was not significant ($P = 0.235$) and thus was not included in the final model. The DM of NES (94 ± 1.2%; n = 49) and RS (92 ± 1.2%; n = 49) were much greater than DM of DBMS (48 ± 1.2%; n = 49) and SBMS (50 ± 1.2%; n = 48; $P < 0.001$). The DM of DBMS and SBMS were similar to those observed by Sorter et al. (2014) in both DBMS and SBMS on d 2 after placement in stalls and by Husfeldt et al. (2012; 55.9 ± 1.9% in digested manure solids, 41.3 ± 3.6% in composted manure solids, 57.2 ± 3.1% in raw manure solids). The DM was lower in winter (66 ± 1.4%) than in summer (73 ± 1.1%), fall (73 ± 1.1%), or spring (72 ± 1.1%; $P < 0.001$). When associations of DM and season were tested within individual bedding types, DM did not differ seasonally for either NES or SBMS. Dry matter was lower ($P = 0.041$) for RS in spring (91 ± 0.9%; n = 56) than in summer (94 ± 1.0%; n = 52) or fall (94 ± 1.0%; n = 52) and did not differ from DM in winter (91 ± 1.2%; n = 35); however, the magnitude of the difference (3.0%) was small. Bedding DM for DBMS was lower in winter than in other seasons (38 ± 3.0%; $P = 0.004$), but did not differ among spring (49 ± 2.3%), fall (50 ± 2.4%), or summer (52 ± 2.4%). The >10 percentage point difference in DM for DBMS in the winter compared with other seasons is much greater than the difference found among seasons for NES.

Unused Bedding. Log_{10} colony-forming units per gram of bacterial counts in unused NES were 2.20 ± 1.56, 0.79 ± 1.27, 0.24 ± 0.76, and 1.71 ± 1.59 for total gram-negative bacteria, coliforms, *Klebsiella* spp., and SSLO, respectively. Log_{10} colony-forming units per gram of bacterial counts in unused RS were 4.52 ± 0.78, 1.49 ± 1.89, 1.57 ± 2.23, and 2.85 ± 1.79 for total gram-negative bacteria, coliforms, *Klebsiella* spp., and SSLO, respectively. Log_{10} colony-forming units per

gram of bacterial counts in unused manure solids were 5.82 ± 2.17, 3.36 ± 1.36, 2.88 ± 1.27, and 4.27 ± 1.70 for total gram-negative bacteria, coliforms, *Klebsiella* spp., and SSLO, respectively.

Teat Skin. Weekly composite PRSWAB (n = 49) and POSWAB (n = 49) were analyzed for teats of cows bedded with each bedding type. One PRSWAB sample of teats of cows bedded with DBMS had no bacteria recovered. Four, 3, 6, and 2 POSWAB samples had no bacteria recovered from teats of cows bedded with NES, RS, DBMS, and SBMS, respectively. The proportion of POSWAB samples with no recoverable bacteria did not differ by bedding type ($P = 0.546$; Fisher's exact test). The number of coliform and *Klebsiella* spp. bacteria cultured from most (88 to 98%) POSWAB were below our limit of detection; thus, no statistical analysis was performed for these organisms (Table 2). Across all bacteria and bedding types, the mean number of bacteria recovered from PRSWAB (cfu/swab) were approximately 2 to 3 logs less than the corresponding number of bacteria in bedding (cfu/g; Table 3). The interaction between bedding type and season did not remain in any of the PRSWAB or POSWAB models.

The fewest total numbers of gram-negative bacteria were recovered from PRSWAB of cows bedded with NES, with 4, 8, and 19 times as many total gram-negative bacteria recovered from PRSWAB of cows bedded SBMS, RS, and DBMS, respectively, as of cows bedded with NES ($P < 0.001$; Table 3). When stratified by season, the numbers of total gram-negative bacteria recovered from PRSWAB were lesser in the summer and fall, and greater in the spring and winter ($P < 0.001$; Table 4). However, the differences in numbers of recovered bacteria were relatively minor with only 0.54 $\log_{10}$ cfu/swab of difference from least to greatest. Meaningful quantities of coliform and *Klebsiella* spp. bacteria (with median >0) were recovered only from PRSWAB of cows bedded with DBMS (Table 3). The number of coliform and Klebsiella bacteria cultured from most (74 to 96%) PRSWAB obtained from teats of cows in pens containing NES, RS, and SBMS were below our limit of detection (Table 2), indicating low levels of exposure to these potential mastitis pathogens when measured as teat skin population. Meaningful (with median >0) numbers of coliform species were only recovered from PRSWAB in the summer (Table 3). Very few total gram-negative bacteria were recovered from POSWAB with median values <200 cfu/swab recovered from teats of cows on any bedding type.

Large numbers of SSLO were recovered from PRSWAB of cows on all bedding types, with the greatest numbers recovered from teats of cows bedded with SBMS and RS, intermediate numbers from teats of cows bedded with NES, and the least numbers from teats of cows bedded with DBMS ($P < 0.001$; Table 3). When stratified by season, the numbers of SSLO recovered from PRSWAB were lower in the summer and fall, and greater in the spring and winter ($P < 0.001$; Table 4). However, the differences in numbers of recovered bacteria were relatively minor, with only a 0.70 $\log_{10}$ cfu/swab difference from least to greatest. Few SSLO were recovered from POSWAB. Median numbers of SSLO recovered from POSWAB were 829, 3,040, 1,106, and 6,219 cfu/swab from teats of cows bedded with NES, RS, DBMS, and SBMS, respectively. With the exception of similar numbers in DBMS ($P = 0.311$), as compared with quantities of SSLO, fewer total gram-negative bacteria were recovered from PRSWAB ($P < 0.001$; Table 3).

Reduction of Teat Skin Bacteria during Premilking Preparation. The average RED did not vary by season for any bacterial group ($P > 0.125$). The average RED were 1.95 and 1.51 log units for total gram-negative and streptococcal bacteria, respectively, and did not differ among bedding types ($P > 0.286$; Table 5). The greatest RED of coliform bacteria was found on teats of cows bedded with DBMS and least RED on teats of cows bedded with SBMS and RS ($P < 0.001$). Greater RED of *Klebsiella* spp. was found on teats of cows bedded with DBMS and RS than on teats of cows bedded with NES or SBMS ($P < 0.001$).

Table 5. Reduction in the number of bacteria on teat skin from premilking preparation Log_{10}(cfu/prepreparation swab + 1) − Log_{10}(cfu/postpreparation swab + 1), mean (SE)

Bedding type	Gram-negative	Coliform	*Klebsiella* spp.	SSLO[1]
Deep-bedded new sand	2.1 (0.21)	2.0^{ab} (0.29)	−0.7^{b} (0.48)	1.6 (0.17)
Deep-bedded recycled sand	2.0 (0.19)	1.8^{b} (0.21)	2.2^{a} (0.19)	1.5 (0.17)
Deep-bedded manure solids	2.0 (0.19)	2.6^{a} (0.16)	2.3^{a} (0.12)	1.7 (0.17)
Shallow-bedded manure solids[2]	1.7 (0.19)	1.4^{b} (0.26)	0.6^{b} (0.39)	1.2 (0.17)

[a,b]Means within a column with different superscripts differ among bedding types (Tukey adjusted $P < 0.05$).

[1]Streptococci and streptococci-like organisms isolated on modified Edwards medium (Oxoid, Basingstoke, UK; supplemented with colistin sulfate and 5% plasma and oxolinic acid as per Hogan et al., 2007).

[2]Over foam-core mattresses.

Correlations of Bacterial Populations in Bedding and on Teat Skin. The only significant within bedding type correlations were of numbers of coliform (r = 0.45; $P = 0.001$) and *Klebsiella* spp. (r = 0.59; $P < 0.001$) in bedding and recovered from PRSWAB in DBMS. When results from all bedding types were pooled, significant ($P < 0.001$) positive ranked correlations were noted between the number of bacteria in bedding and recovered from PRSWAB for total gram-negative bacteria (r = 0.33), coliforms (r = 0.41), *Klebsiella* spp. (r = 0.45), and SSLO (r = 0.35).

DISCUSSION

This study was conducted at a research facility that was designed to study differences among various bedding materials in 4 identical pens containing randomly assigned lactating cows (USDA-ARS, 2011). The cows had access to freestalls similar to housing of cows on many commercial dairy farms throughout the United States. Most large dairy farms in temperate climates house lactating cows in freestalls and a recent study reported that 99.6% of larger WI dairy farms used freestalls for lactating cows (Rowbotham and Ruegg, 2015). Milk production of cows at the University of Wisconsin research facility was similar to US milk production (32.5 kg/d), indicating that nutritional management and genetic potential of the research herd was similar to commercial dairy farms (USDA-NASS, 2014). However, the distribution of parities in this experimental herd was not typical of US dairy farms (which contained an average of 35% primiparous cows; Hare et al., 2006) and may have influenced the results. At the beginning of this study, our herd contained 85% primiparous Holsteins. During the study, 84% of replacements were also primiparous Holsteins. Because the research station is adjacent to a University of Wisconsin heifer-raising facility, as cows reached the end of their lactation they were typically replaced with primiparous animals. The predominance of first-parity animals in the research herd probably resulted in decreased contact of teats to bedding due to smaller udders with lesser udder depth, which may have resulted in lower numbers of bacteria recovered from teat swabs. No quench solution (Fox, 1992) was used as we were measuring potential exposure of teats to bacteria in bedding. The failure to use a quench solution in the presence of potential iodine residue from teat dipping may have resulted in fewer bacteria; however, this potential error would not have introduced bias as it was evenly distributed among treatments.

Decreasing availability and increasing costs of traditional dairy cattle bedding materials (such as straw and sawdust) have promoted increased interest in use of sand or recycled manure solids as bedding (Husfeldt and Endres, 2012; Husfeldt et al., 2012). Sand (45%), sawdust (21%), straw (14%), and manure (7%) are the 4 most common bedding materials in US freestall barns, with 87% of freestall barns using these bedding types (USDA, 2008). Of 325 large WI dairy farms, bedding materials used in freestalls included fresh (58%) or recycled (10%) sand, organic materials (primarily wood products on top of mattresses; 22%), and manure products (10%; Rowbotham and Ruegg, 2015). Thus, the types of materials used in the 4 pens were typical of materials used on larger freestall operations in WI.

Washed sand is often considered an ideal bedding material for dairy cattle because it is low in moisture content and contains few nutrients to support bacterial growth (Hogan and Smith, 2012). However, the recurring expenses of purchasing sand and handling sand laden manure has increased interest in recycling sand. By recycling sand to reuse as bedding, the total tonnage of DM in the manure stream are reduced. However, on some dairy farms, sand is incompatible with manure management, making organic bedding materials attractive to some producers (Hogan et al., 1990; Husfeldt and Endres, 2012). Interest in using recycled manure solids for bedding has increased, especially in the Midwest (Husfeldt and Endres, 2012), partially due to the recent introduction of on-farm anaerobic manure digestion (Godden et al., 2008). Farmers using recycled manure solids, in contrast to other organic bedding types, reduce the total amount of nutrients which become part of the manure stream due to no net addition of nutrients in the form of bedding, thus increasing potential compliance with environmental regulations (United States Congress, 1972). However, whereas herds with digesters can produce milk that meets SCC standards, recent research (Rowbotham and Ruegg, 2015) indicates that maintaining udder health (especially managing clinical mastitis) can be a challenge for herds that use digesters as a source of bedding material. In WI, the use of manure-based bedding has been associated with increased SCC, increased proportions of cows with discarded milk (due to treatment), and an increased prevalence of nonfunctioning mammary glands; these indirect effects may reduce productivity and overall herd profitability (Rowbotham and Ruegg, 2015).

In our study, bedding and teat swab samples were frozen for 4 to 25 d before undergoing laboratory analysis. Freezing of samples has been used for decades as the primary method to prevent incubation and bacterial growth during storage and transportation of samples in the absence of better methods. Homerosky and Hogan (2015) reported no difference in the number of gram-negative, coliform, *Klebsiella* spp., or streptococci bacteria in fresh sand or recycled manure solids bedding

among samples frozen for 7 to 21 d. They did, however, report reductions between numbers of coliform bacteria in recycled manure solids and total numbers of gram-negative bacteria in sand and recycled manure solids when comparing fresh and frozen samples. As compared with culture of fresh samples, freezing all of our samples for a short period likely resulted in recovery of fewer total gram-negative bacteria from all bedding types and fewer coliform bacteria from samples of NES and RS. However, all samples were handled uniformly and freezing would not have influenced the comparisons reported in our study. Prior to abstract publication by Homerosky and Hogan (2015), the effects of freezing of bedding samples on observed bacterial populations had not been reported. Because of small sample sizes in their study, potentially resulting in type II error, comparisons of different groups of bacteria among frozen bedding samples should be interpreted with caution.

In recent studies, the most common pathogens recovered from milk samples obtained from cows with clinical mastitis on large WI dairy herds have been *Escherichia coli*, environmental streptococci, and *Klebsiella* spp. (Pinzón-Sánchez and Ruegg, 2011; Oliveira et al., 2013). Streptococci and coliforms do not survive on teat skin for long periods of time, therefore streptococci and coliforms isolated from teat skin are primarily from recent contamination, likely through contact with bedding (Hogan and Smith, 2003, 2012). It is possible that SSLO colonize teat skin longer and more effectively than coliforms, which may also influence infection rates. Coliforms are normal flora of the cow gastrointestinal tract and manure may contaminate bedding with coliform bacteria (Hogan and Smith, 2003). Environmental streptococci have been isolated from many fomites in the cow environment including bedding, feces, and feed (Bramley, 1982; Hogan and Smith, 2012). Once inoculated with bacteria and contaminated with manure (a nutrient source for bacteria), bedding has the ability to maintain and contribute to growth of bacterial populations. Coagulase-negative staphylococci, minor mastitis pathogens, are considered normal flora of teat skin and opportunistic bacteria that sometimes cause mastitis (Devriese and Dekeyser, 1980; Ruegg and Dohoo, 1997). Whereas some species of CNS may live in the environment, the main species causing mastitis are specifically adapted to live on teat skin and in the udder (Pyörälä and Taponen, 2009). It is for this reason that CNS have not been regularly cultured as part of mastitis-related bedding trials and were not included in our study.

Many previous studies of bacterial populations in bedding have measured populations per unit mass of bedding. All but 2 studies referenced in this manuscript used this methodology, the exceptions measuring bacterial populations per unit volume of bedding (Godden et al., 2008; Husfeldt et al., 2012). Hogan et al. (1999) reported bacterial populations per milliliter of solution, but diluted an equal mass of each bedding type in the same volume of diluent, thus calculating populations per unit mass and reporting per unit volume. We chose to measure and report bacterial populations per unit mass for 2 reasons. First, for consistency with existing literature, and second to control for variable compaction of compressible bedding materials. Manure solids are compressible and the question of exposure to volumes of bedding as compared with masses of bedding should be addressed. The literature does not report if a cow is exposed to compressible bedding as fluffed in the stall, as compressed by BW from lying behavior, or as compressed by concentrated weight as concentrated under the hoof of a standing animal.

The ability of bedding to support bacterial growth varies among bedding types and may be dependent upon physical, biochemical, or nutritional properties of the bedding (Godden et al., 2008). Moisture, adequate nutrients, and sufficient temperatures are basic growth requirements for bacteria. Greater percent moisture and content of OM found in organic types of bedding as compared with inorganic types of bedding are usually associated with greater numbers of gram-negative bacteria, coliforms, *Klebsiella* spp., and streptococci (Hogan et al., 1989). Researchers conducted an in vitro study of sterilized bedding (NS, RS, digested manure solids, shavings) inoculated with *Klebsiella pneumonia* or *Enterococcus faecium* (Godden et al., 2008). After 72 h, numbers of *Klebsiella pneumonia* were 1 and 2 log greater in RS and digested manure, respectively, than in NES. Digested manure solids and RS were able to maintain populations of *Enterococcus faecium* for 72 h; however, NES was not, with bacteria entering a death phase shortly after inoculation. The lesser ability of NES as compared with RS and digested manure solids to support growth of potential gram-negative mastitis pathogens supports the recommendation of the use of washed sand as ideal bedding for the control of these organisms (Hogan and Smith, 2012). Because bacteriological results of bedding cultures are usually reported as colony-forming units per unit mass, moisture is reported as a percentage of mass, and great variation can occur among the density of bedding materials, there is opportunity to study the relationship among bedding density, moisture content, and bacterial populations not explored in the present study. A small change in the percent moisture of a relatively dense bedding material, such as sand, may represent a greater change in total water per unit volume than a larger percentage change in moisture content of a less-dense bedding, such as manure solids. As discussed earlier, all relationships in the current study are reported as a relation to mass,

not volume, to avoid potential bias from inconsistent measurement of highly compressible bedding materials (DBMS, SBMS). One oversight of our study was the omission of measuring the OM content of bedding materials, which may also be an important factor in the ability of bacteria to grow in bedding.

In our study, we found coliform bacteria (*Escherichia* spp., *Klebsiella* spp., and *Enterobacter* spp.; Fairchild et al., 1982) to be only 2 to 7% of total gram-negative bacteria in bedding. Other gram-negative bacteria of environmental origin known to cause mastitis include *Bacillus* spp., *Serratia* spp., *Pseudomonas* spp., and *Proteus* spp. (Smith and Hogan, 2008). In a recent study of large WI dairy herds which characterized clinical mastitis, the 3 most commonly isolated pathogens were *E. coli* (47% of gram-negative isolates), environmental streptococci (47% of gram-positive isolates), and *Klebsiella* spp. (19% of gram-negative isolates; Oliveira et al., 2013). In another recent study, the most common isolates were environmental streptococci (63% of gram-positive isolates), *E. coli* (33% of gram-negative isolates), and *Klebsiella* spp. (26% of gram-negative isolates; Pinzón-Sánchez and Ruegg, 2011). Because environmental streptococci and coliform bacteria are the most commonly isolated clinical mastitis pathogens on modern dairy farms, they are the bacteria most commonly cultured using selective and differential media when researching potential mastitis pathogen populations in bedding and on teat skin.

Previous researchers have observed greater numbers of bacteria in organic bedding than in inorganic bedding (Fairchild et al., 1982; Janzen et al., 1982; Hogan et al., 1989). Our finding of fewer total gram-negative bacteria in NES and RS than in DBMS and SBMS is in agreement with these previous observations. Because streptococci have been isolated from vulva, lips, nares, teats, and the mammary gland as well as soil, feces, bedding, and feed in the cow's environment (Bramley, 1982; Hogan and Smith, 2012), we expected to recover them from all bedding types. Least squares means numbers of SSLO in bedding observed in our experiment were similar to mean $\log_{10}$ numbers of streptococci in beddings grouped as organic (7.5) and inorganic (6.8) and in recycled manure solids (7.5) from previous research (Hogan et. al., 1989; Todhunter et al., 1995). Even though we observed statistically significant differences in the numbers of SSLO among bedding types, large numbers of SSLO (median $>8.6 \times 10^6$) were recovered from all bedding types, indicating considerable potential exposure to this pathogen regardless of bedding type. This implies that selection of bedding may not be beneficial for controlling exposure to SSLO because they are ubiquitous in the environment. Streptococci and streptococci-like organisms are normally associated with increased SCC and can cause persistent subclinical infections, which may exhibit sporadic clinical episodes; thus, cows exposed to sand-based bedding are still at considerable risk of exposure to SSLO, which may initially manifest as increased SCC rather than increased clinical cases.

Both depth and frequency of replacement of manure solids bedding play important roles in reducing bacterial populations in manure bedding. Sorter et al. (2014) compared bacterial populations of DBMS and SBMS based on frequency of removal and replacement in a freestall barn. Each day, all bedding was removed from the back one-third of SBMS stalls and replaced with bedding from the fronts of the stalls. Fecal matter was removed from the DBMS stalls once daily and new bedding was added to all stalls weekly. In agreement with our findings, Sorter et al., (2014) reported fewer numbers of coliforms, including *Klebsiella* spp., in SBMS than in DBMS and greater numbers of SSLO in DBMS than in SBMS. However, in contrast to our findings of 1 log greater numbers of total gram-negative bacteria in DBMS as compared with SBMS, Sorter et al., (2014) found no difference in total numbers of gram-negative bacteria. Thus, shallow bedding of manure solids over mattresses may be as effective as daily removal of and replacement of the same bedding for the control of streptococcal, coliform, and *Klebsiella* spp. populations; however, more intensive bedding management may be required to manage total gram-negative bacterial populations.

Seasonal variation in bacterial populations of bedding is mediated by changes in ambient temperature and humidity, important factors which affect growth of bacteria (Godden et al., 2008). Two previous studies have quantified the relationships between bacterial populations in bedding during different seasons. Kristula et al. (2005) collected samples of clean or recycled sand bedding during a single week per farm from 12 commercial dairy farms in winter (February and March) and from 11 farms during summer (July through September). They found no differences in total number of gram-negative bacteria, coliforms, *Klebsiella* spp., or streptococci between samples of NES and RS within either season. They did not report analysis of seasonal differences in numbers of bacteria. Kristula et al. (2005) reported significant variation in numbers of bacteria among samples, but were unable to associate these differences with bedding type due to uncontrolled differences among facility types. We were able to find significantly greater numbers of bacteria in RS as compared with NES, which were not observed in the earlier study, probably due to larger sample size and controlling for the effects of farm by using a single facility. As measured on d 3 after placement of bedding into stalls,

the coefficient of variation of bacteria in bedding in Kristula et al. (2005) ranged from 39 to 168%, with all values >100% except total gram-negative bacteria in RS; our coefficient of variation was between 7 to 49%, exceeding 23% only for *Klebsiella* spp.

Another study compared bacteria populations in bedding among seasons by collecting samples monthly for 1 yr from 9 commercial dairy farms (Hogan et al., 1989). The farms used sand (n = 1), crushed limestone (n = 1), sawdust (n = 3), chopped straw (n = 3), or sawdust during the summer and sand for the remainder of the year (n = 1). In agreement with our findings Hogan et al. (1989) reported greater numbers of gram-negative bacteria, coliforms, *Klebsiella* spp., and *Streptococcus* spp. in organic versus inorganic bedding. Hogan et al., (1989) observed differing seasonal effects for various bacteriological groupings depending upon the type of bedding. Consistent with our findings of consistently increased numbers of SSLO, those authors reported no seasonal differences in numbers of streptococci for any bedding type and reported greater total numbers of gram-negative bacteria, including coliform in summer and fall than in winter and spring in chopped straw, which were consistent with our results. Hogan et al. (1989) reported no differences in numbers of any bacterial group among seasons for inorganic bedding; however, only 2 farms used inorganic bedding throughout the study and 1 additional farm did for 3 of 4 seasons. Again, our experiment may have been able to find differences not previously reported by controlling for facility differences. We were also able to sample all bedding types on the same day each week (n = 49), which would not have been practical when sampling bedding from many different farms.

Although the DM of sand was much greater than that of manure solids, the differences may be attributable to physical or chemical properties, because sand traps moisture between particles whereas manure solids trap and also absorb moisture. For this reason, DM content was compared within bedding types. We noted very little variation in the DM content of sand; however, the sand was very dry with both NES and RS having over 90% DM. The only large difference in bedding DM among seasons was observed in DBMS, with DM in winter more than 10 percentage points less than in other seasons. In winter, the sidewall curtains of the freestall barn were closed and fans were rarely used, thus less air circulation occurred in winter than in other seasons, possibly leading to increased moisture content in DBMS in the winter as compared with other seasons. Dry matter content of both DBMS (48 ± 1.2%) and SBMS (50 ± 1.2%) was greater than the average DM observed of separated raw manure used as bedding for dairy cattle on farms in the Midwest (42.8%; n = 13; Husfeldt et al., 2012). The bedding samples in our study were collected 3 d after placement in the stalls and, although it is unknown how many days after application of the bedding Husfeldt et al. (2012) collected samples, if the bedding had remained in the stalls longer than 2 to 3 d it would probably have contained less moisture (in contrast to more moisture as we observed). The DM of DBMS and SBMS that we observed were similar to those observed by Sorter et al. (2014) in both DBMS and SBMS on d 2 after placement in stalls. Sorter et al. (2014) observed increased DM content throughout the week following placement into stalls. The greater density of sand as compared with manure solids dictates that a change in the percent moisture of sand represents a greater change in the total mass of water than the same percentage change in the moisture content of manure solids.

To explore associations in numbers of bacteria in bedding and on teat skin among bedding types, we used Tobit regression models. Tobit models are used to estimate linear relationships where there is censoring of the response variable for some samples. In our case, when numbers of bacteria were less than our lower limit of detection, the response variable was censored and reported as no growth. Among bedding samples, only *Klebsiella* spp. numbers were below our limit of detection for >10% of samples. Among PRSWAB, from 4 to 96% of samples were below our limit of detection, and among POSWAB, as many as 98% of samples were below our limit of detection; this allowed us to make accurate comparisons among bedding types with Tobit regression, which would not have been possible using linear regression.

In agreement with our findings, previous researchers have consistently reported greater numbers (0.5–1.5 log units) of bacteria on teats of cows bedded with organic materials than on teats of cows bedded with inorganic materials (Fairchild et al., 1982; Janzen et al., 1982; Zdanowicz et al., 2004), with the exception of one study in which 1 log greater numbers of streptococci were reported from teats of cows bedded with sand as compared with sawdust (Janzen et al., 1982). Most previous researchers collected and enumerated bacteria on teat swab samples from individual teats or composite swab samples of individual cows. Because our experimental unit was the pen, we collected 1 swab from a single teat of each cow, which were accumulated into 1 composite sample per pen similar to methods used by Kristula et al. (2008). Our dilution methods allowed us to enumerate bacteria as $\log_{10}$ colony-forming units per teat swab, consistent with previous reporting (Rendos et al., 1975; Hogan and Smith, 1997; Zdanowicz et al., 2004) equating each swab to one teat; this was in contrast to other researchers who either reported numbers of bacteria in

$\log_{10}$ colony-forming units per milliliter of diluent (Fairchild et al., 1982; Hogan et al., 1990; Proietto et al., 2013) or reported results without a defined unit for the denominator (Janzen et al., 1982; Kristula et al., 2008).

Understanding both environmental exposure (measured as bacterial populations in bedding) and the transfer of those bacteria to the teat skin is important because bedding is a primary reservoir for environmental pathogens and may be in direct contact with teats for 12 to 14 h/d (Tucker and Weary, 2004; Cook et al., 2005; Hogan and Smith, 2012). Exposure to environmental pathogens found in bedding occurs when teats are exposed to the bedding and bacteria are transferred to the teat skin. Researchers have reported significant positive correlations between bacterial populations in bedding and on teat skin (Hogan and Smith, 1997; Hogan et al., 1999; Zdanowicz et al., 2004); however, 2 of these studies measured correlations after pooling results from all types of bedding (Hogan and Smith, 1997; Hogan et al., 1999). One previous study reported a lack of significant correlations between bedding and teat skin bacterial populations when evaluated within each bedding type (recycled newspaper, wood shavings, pelleted corn cobs), but reported significant correlations when all results were pooled (Hogan et al., 1990). One reason correlations might have been lacking is due to variations with bedding types when measured across multiple farms. Our study found a similar relationship, with correlations between numbers of bacteria in bedding and recovered from PRSWAB positive and significant only for numbers of coliform and *Klebsiella* spp. within DBMS when stratified by bedding type. Consistent with prior results, we found significant positive correlations between the number of bacteria in bedding and on PRSWAB results that were pooled for all bedding types. In our study, bacteria counts below our limit of detection were coded as missing data points for PROC CORR and therefore excluded from analysis. Sample size for correlations when all bedding types were combined were 179, 74, 45, and 193 for total gram-negative bacteria, coliforms, *Klebsiella* spp., and SSLO, respectively. When correlations were measured between bedding and teat swab counts within bedding types, samples sizes were between 39 and 47 for total gram-negative bacteria, between 10 and 24 for coliform bacteria, between 0 and 32 for *Klebsiella* spp., and between 46 and 49 for SSLO. Because of small sample sizes within bedding types, our inability to detect correlations was not unexpected. Because correlations between numbers of bacteria in bedding and on PRSWAB differ among bedding types, it is important to measure numbers of teat skin bacteria to estimate exposure rather than relying on numbers of bacteria in bedding. Differences may exist in exposure, measured as teat skin bacterial populations, not only among different bedding types but also due to freestall design or bedding maintenance. Addition of inorganic bedding to stalls more than once weekly has been associated with increased bulk milk SCS, and completely removing and replacing nonmanure organic bedding in the backs of stalls at least weekly has been associated with decreased bulk milk SCS (Rowbotham and Ruegg, 2015). Older cows with more pendulous udders might also have greater exposure to environmental pathogens found in bedding than our predominantly primiparous research herd.

In our study, the number of gram-negative bacteria recovered from PRSWAB of teats of cows bedded with NES was 0.65 to 1.3 log units less than numbers recovered from PRSWAB of teats of cows bedded with RS, DBMS, and SBMS. Except for equivalent numbers of coliform bacteria, the number of bacteria recovered from PRSWAB was 0.54 to 0.91 log units greater from teats of cows bedded with RS than from teats of cows bedded with NES. In our study, exposure to total gram-negative bacteria, coliforms, *Klebsiella* spp., and SSLO was greater for cows bedded with RS as compared with cows bedded with NES. Many gram-negative pathogens cause short-duration infections that result in clinical signs (Smith et al., 1985; Hogan and Smith, 2003). The implication for dairy farmers is that greater exposure to gram-negative bacteria may result in greater risk of clinical mastitis as compared with subclinical mastitis.

Similar to the great majority of large WI dairy farms (Rowbotham and Ruegg, 2015), milking technicians at the experimental station always used a complete milking routine that always included forestripping, predipping, drying of teats, and postdipping. Researchers previously have reported 0.5 to 1.3 log units of reduction in total teat bacteria populations associated with premilking teat sanitization (Galton et al., 1984; Gibson et al., 2008). Using various premilking teat disinfectants resulted in 50 to 95% reductions in *Staphylococcus* spp. and *Streptococci* spp. on teat skin and between 0 and 15% reductions in numbers of coliform on teat skin (Gleeson et al., 2009). Our results of 1.4 to 2.1 RED in numbers of gram-negative, coliform, and SSLO bacteria are similar to but numerically greater than previously reported results. One explanation for the greater efficacy of premilking sanitization in our experiment could be the heightened emphasis placed on premilking sanitization at the research station, which is similar to well-managed large dairies in WI. Proper premilking sanitization can successfully reduce teat skin bacterial populations. During milking, bacteria on the teat skin may enter the teat canal, thus the number of bacteria on teat skin at the time of milking machine attachment represents the level of exposure risk to potential mas-

titis pathogens. Proper premilking sanitization, including use of a germicidal predip and drying teats with a clean towel per cow, reduces the number of bacteria on the teat skin (Galton et al., 1984) and thus reduces the potential exposure of the teat canal to these bacteria during milking.

CONCLUSIONS

There were large numbers of SSLO in all bedding types, with the greatest numbers in SBMS. With the exception of DBMS, where numbers of SSLO and total gram-negative bacteria were similar, numbers of gram-negative bacteria were approximately 2 to 3 log lower than numbers of SSLO in bedding. Whereas SSLO were numerous in all bedding types, numbers of gram-negative bacteria were much lower in NES than in other bedding types. Similar to numbers in bedding, large numbers of SSLO were found on teats of cows housed in all bedding types; however, the lowest numbers were recovered from teats of cows in DBMS. The lowest gram-negative bacteria were recovered from teats of cows bedded with NES, and meaningful numbers of coliform and *Klebsiella* spp. were recovered only from teats of cows bedded with DBMS. Large numbers of SSLO found in all bedding types may indicate that the risk of subclinical mastitis is not influenced by bedding type. Overall, these findings indicate that different exposure to potential mastitis pathogens in different bedding materials may alter the incidence rate of clinical mastitis observed; however, bedding is not the only potential source of environmental exposure and more research is required to determine associations among selective bacterial counts from bedding samples and rates of mastitis.

ACKNOWLEDGMENTS

The authors thank the USDA Agricultural Research Service for the use of their facilities, the University of Wisconsin-Madison for the use of their dairy herd, the dairy farm personnel at the Marshfield Agricultural Research Station for their participation in this project, and the Grande Cheese Company (Brownsville, WI) for project funding.

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