Bacterial concentrations in bedding and their association with dairy cow hygiene and milk quality


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Comparison of bacterial counts (BCs) among common bedding types used for dairy cows, including straw, is needed. There is concern that the microbial content of organic bedding is elevated and presents risks for dairy cow udder health and milk quality. The objectives of this study were to investigate: (1) % DM and BCs (Streptococcus spp., all gram-negatives and specifically Klebsiella spp.) in different types of bedding sampled, and to investigate housing and farm management factors associated with % DM and BCs; (2) if bedding type was associated with hygiene of cow body parts (lower-legs, udder, upper-legs and flank) and housing and management factors associated with hygiene and (3) bedding types associated with higher BCs in cow milk at the farm level and bulk tank milk and management factors that were associated with highest BCs. Seventy farms (44 free-stall and 26 tie-stall) in Ontario, Canada were visited 3 times, 7 days apart from October 2014 to February 2015. At each visit, composite samples of unused and used bedding were collected for % DM determination and bacterial culture. Used bedding samples were collected from the back third of selected stalls. Data were analyzed using multivariable linear mixed models. Bedding classification for each farm were: new sand (n = 12), straw and other dry forage (n = 33), wood products (shavings, sawdust; n = 17) and recycled manure solids (RMSs)-compost, digestate (n = 8). In used bedding, across all bedding samples, sand was driest, compared to straw and wood, and RMS; higher % DM was associated with lower Streptococcus spp. count. Streptococcus spp. and all Gram-negative bacteria counts increased with increasing days since additional bedding was added. Gram-negative bacteria counts in used bedding varied with type: RMS = 16.3 ln colony-forming units (cfu)/mL, straw = 13.8 ln cfu/mL, new sand = 13.5 ln cfu/mL, and wood = 10.3 ln cfu/mL. Klebsiella spp. counts in used bedding were lower for wood products (5.9 ln cfu/mL) compared to all other bedding types. Mean cow SCC tended to be higher on farms with narrower stalls. Farms with mattress-based stalls had a higher prevalence of cows with dirty udders compared to those using a deep bedding system (often inorganic sand). Wider stalls were associated with lower bulk milk bacteria count. Lower % DM of used bedding was associated with higher bulk milk bacteria count. In conclusion, bedding management may have a profound impact on milk quality, bacterial concentrations in the bedding substrates, and cow hygiene.

Keywords: bacterial count, housing, cleanliness, dry matter, management

Implications

This study includes estimates of bacteria counts in various dairy bedding types, including straw, which is widely used in the dairy industry, wood products, sand and recycled manure solids. This study highlights the association of higher percent dry matter in bedding with reduced bedding bacteria counts as well as with greater bulk tank milk quality. Also, the results emphasize the benefits associated with frequent addition of bedding, including lower bacterial count in used bedding, and improved cow hygiene. Finally, this research pinpoints factors of housing, milking systems and stall design associated with cow hygiene. This study provides practical information for producers and research focus areas regarding farm management practices associated with milk quality and cow hygiene.

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Introduction
Mastitis is a very costly disease to the dairy industry. A recent study assessing farm-level mastitis associated costs on Canadian dairy farms concluded that the median cost of mastitis was $662 CAD/cow per year (Aghamohammadi et al., 2018). Environmental bacteria, including Escherichia coli, Klebsiella spp. and Streptococcus uberis, are a common cause of clinical mastitis in dairy cows, according to data from 59 dairy farms in Southern Ontario, Canada (Levison et al., 2016). Another study reported that the most predominant pathogen causing clinical mastitis was E. coli (on farms with a low bulk milk somatic cell count (SCC)), and besides the contagious pathogen Staphylococcus aureus, other notable pathogens in the studied farms were E. coli and Streptococcus dysgalactiae (on farms with a high milk SCC; Barkema et al., 1998). According to the Canadian Bovine Mastitis Research Network (CBMRN), the primary pathogens cultured from milk samples taken from cows with clinical mastitis cases (expressed as a % of all cases) in the province of Ontario were: 15.4% S. aureus, 11.6% E. coli, 9.6% Streptococcus spp., 4.5% CNS, 3.2% S. dysgalactiae, 1.8% S. uberis and 0.9% Klebsiella spp. (CBMRN, 2010).

Dirty or wet bedding can become heavily contaminated with these bacteria and act as a source of intramammary infections (IMIs) that may result in (sub) clinical mastitis (e.g. Hogan and Smith, 2012). It is, therefore, important to keep bedding clean and, as a result, reduce exposure to primary agents that may cause environmental mastitis (Hogan and Smith, 2012). In a study at a single facility, following 1234 quarters from 309 cows, all reported cases of clinical mastitis cases (n = 111), with the exception of a single case, were caused by environmental pathogens (Rowbotham and Ruegg, 2016a). Furthermore, these same authors noted that environmental Streptococci, E. coli and Klebsiella spp. were identified in 50% of culture-positive cases of clinical mastitis and were also found in cultures of bedding and teat swab samples (Rowbotham and Ruegg, 2016a).

Indoor housed dairy cattle spend an average of 10 to 13 h/day lying down (Vasseur et al., 2012) and during that time bacteria can be transferred from the lying surface to the teat (Hogan et al., 1999). Direct exposure to pathogens may, therefore, occur as cows rest (Rowbotham and Ruegg, 2015); this in turn may increase risk of IMI, particularly during times when cows are susceptible to said infections (e.g. DeVries et al., 2012). Reducing the bacterial count (BC) of the bedding surface is, therefore, a key step in mastitis control.

Materials used as bedding for dairy cows are often classified as either organic (i.e. straw and wood products) or inorganic (i.e. sand) (Hogan et al., 1989). Although straw is a common bedding material in some regions, weather conditions and location directly impact the availability of straw (Manninen et al., 2002). Sawdust and other wood products are also used as bedding, but straw availability and cost have forced producers to look for alternative materials (Husfeldt et al., 2012). Green box compost made from biodegradable waste from households is another bedding option that has been used (Groot Antink, 2009). Due to advanced technology in separation and anaerobic digestion, in addition to the large amounts of bedding that can be obtained from recycled manure solids (RMSs), RMS is an alternative bedding type receiving much attention (Godden et al., 2008; Husfeldt et al., 2012). Sand is an inorganic bedding type that is highly recommended (van Gastelen et al., 2011) and widely regarded as the most ideal bedding type for dairy cows. Many researchers (e.g. Zdanowicz et al., 2004; Kristula et al., 2005; Rowbotham and Ruegg, 2016a) have demonstrated the advantage of using clean and dry sand (inorganic bedding) as a way of reducing the growth of bacteria associated with environmental mastitis. However, producers face numerous challenges when attempting to handle, use and dispose of sand, and given the high costs of traditional organic bedding sources efforts have focused on identifying alternative bedding sources (Husfeldt et al., 2012). Like sand, RMS may be placed in deep-bedded stalls; large amounts of bedding are known to maximize stall usage, reduce lameness and hock lesions (Tucker and Weary, 2004). However, RMS has a higher BC compared to sand (Rowbotham and Ruegg, 2016b) and shavings (Godden et al., 2008). Bradley et al. (2018) concluded that despite the elevated BC in RMS, it does not necessarily result in higher BCs in bulk tank milk as compared to sand and sawdust. To our knowledge, no recent study has included a comparison between the BC of RMS and straw, which is also commonly used as bedding on dairy farms. Producers that only have access or can only utilize organic bedding sources would be better informed if there was updated information on which bedding types had higher BCs and bedding management practices that are associated with cow hygiene and milk quality.

The objectives of this study were to investigate: (1) the %DM and BCs (Streptococcus spp., all gram-negatives, and specifically Klebsiella spp.) in the different types of bedding sampled, and to investigate housing and farm management factors associated with bacterial % DM and BCs; (2) if bedding type was associated with hygiene of cow body parts (lower legs, udder, upper legs and flank) and housing and management factors associated with hygiene and (3) bedding types associated with higher BCs on cow milk at the farm level and bulk tank milk and management factors that were associated with higher BCs. It was hypothesized that organic-based bedding, particularly manure-based bedding options, would have the very low % DM compared to the rest of the bedding types sampled. It was also hypothesized that organic-based bedding, particularly manure-based bedding options, would be associated with a higher BC and higher herd SCC. Additionally, we hypothesized that management practices, such as frequency of addition of new dry bedding and frequency of manure removal, would be associated with cow hygiene and milk quality.

Bacterial count in different bedding types
Material and methods

Farm selection
A cross-sectional study was conducted on 75 dairy farms in Ontario, Canada. Farms were visited three times, 7 days apart, from October 2014 to February 2015, during the fall/winter season. Farms were recruited through an email sent by Lactanet (Guelph, ON, Canada) to producers located within 150 km of the University of Guelph, Kemptville Campus (Kemptville, ON, Canada). Participation of producers in the study was voluntary. Producers were asked to contact the researcher directly, to obtain more information about the project and then confirm interest in enrolling in the study. Specific criteria for selection of farms included: dairy herd improvement (DHI) participation and having lactating cows housed in a free-stall, tie-stall or bedded-pack barn with bedding on the stalls/lying area. Sample size was determined a priori through power analysis using the Power Analysis and Sample Size software program (PASS, Kaysville, UT, USA; Hintze, 2008). Estimate of variation for the primary response variable, bedding bacterial content, was based on that previously reported (Hogan et al., 1999; Hogan and Smith, 1997; Zdanowicz et al., 2004). An estimated sample size of 15 farms per bedding classification type (60 farms total) was determined sufficient to detect a difference of 15% in bedding BCs. A greater number of farms were recruited for participation to try reach our desired sample size per bedding type. If farms were further than the distance of 150 km from campus or used a bedding type that was unlikely to be used for milking cows in multiple farms, to meet the aimed sample size per bedding classification, those farms were then removed from the list of farms to visit. Two producers that expressed their interest in joining the study, but their farms were located too far from campus, were not included in the study. Only eight farms with RMS were recruited, however, even with that sample size we estimated that we had sufficient power to detect a difference of 7.3% in bedding BCs. Animal use and study design were approved by the University of Guelph's Animal Care Committee (AUP#3140) and Research Ethics Board (REB#14JN019), respectively, and animal use complied with the guidelines of the Canadian Council on Animal Care (2009).

Farm data collection
Producers were interviewed by a trained researcher about routine management practices for lactating cows on the first visit to each farm, including information about the bedding, housing management practices and udder health. Producers provided researchers with details on frequency of bedding applied, including time of last raking/cleaning, type of bedding application (whole stall, back half) and whether bedding conditioners were applied to the bedding. Type of bedding and the base material of lying stalls were recorded by the researcher. Producers were asked for the date when additional bedding was last added to the stalls on each visit (total three visits).

Stall dimensions (including stall width, length and neck rail position) were recorded in one of the three visits, using methodology adapted from von Keyserlingk et al. (2012). For free-stall barns, two stalls from each row in each pen were measured. For tie-stall barns, 2 (but not consecutive) stalls of every 20 stalls in each row of stalls were measured (minimum of 4 stalls per farm). Overall stall dimensions (mean and SD) for each farm were calculated. Temperature and relative humidity of the environment in the 7 days prior to each farm visit were obtained from Environment Canada (Ottawa, ON, Canada) recordings at the closest local weather station (Schüller et al., 2013).

Animal selection
At each farm, 25% of the cows in each lactating pen (free-stalls) or row of stalls (tie-stalls) in farms with >160 cows, or a minimum of 40 cows/farm in those farms with <160 cows, were randomly selected (from each area of the pen, in all milking cow pens) and scored for hygiene. If a farm had <40 cows lactating at the time of the study, all lactating cows were scored (n = 8). The same cows were scored on each of the three visits (n = 2770 cows). Percentage of cows selected was based on recommendations made by Cook and Reinemann (2007) regarding the percentage of animals sampled from a given farm that would be representative of the farm. In brief, the scoring system involved scoring 25% of the cows in each pen and to scoring the amount of manure present in lower legs, upper legs and flank, and udder of each cow scored. Systematic random sampling was used to select individuals by only including every nth cow, based on the number of cows needed relative to the size of each pen. This ensured that cows were selected proportionately from all parts within a pen (i.e. those lying down, feeding, standing idly, etc; King et al., 2016). In cases of farms housing milking cows in more than one pen, a proportionate number of cows per pen were selected to ensure that a representative and random sample was achieved. Selection of individual cows for hygiene scoring on the first farm visit was restricted to those cows expected to stay in the lactating pens for the duration of the study (15 days) which included three visits in total, every 7 days. Data from 93 cows were not included in the analyses as those animals were sold (n = 33 cows), dried (n = 59) or euthanized (n = 1). Udder, lower legs and upper legs/flank were each scored on a 4-point hygiene scale (1 = very clean to 4 = very dirty) (Cook and Reinemann, 2007), and the percentage of cows with poor hygiene (scores 3 and 4) was calculated per farm and used for statistical analysis (Schreiner and Ruegg, 2003). Two trained researchers scored the cows, with each researcher scoring the same cows at each visit.

Bedding samples
At each farm visit, duplicate composite samples of the unused and used bedding material were collected in each of three visits in total, every 7 days, to investigate % DM and perform bacterial culture. Time during the day was not specific (−0800 to 1700 h) and could vary between visits; however, variability was the same for all bedding types given that farms visits were not dependent on bedding type.
Unused bedding samples were collected from separate sections of the storage piles on each farm. Each composite sample taken from the pile contained nine grab samples, each approximately the size of a golf ball (4 cm in diameter), with approximately half representing the surface (not deeper than 5 cm) and the remaining from ~15 cm into the pile. When more than one pile of new bedding was available, the same collection procedure was repeated on all piles. Samples were placed in a 20 l pail, thoroughly mixed, and two smaller composite samples, each ~500 cc (two cups of volume), were labelled and stored. Used bedding samples from the cow lying surfaces were collected from the back 1/3 of every 10th stall in each lactating cow pen (free-stall) or row of stalls (tie-stalls). Nine grab samples of bedding were collected from the back 1/3 of each selected stall avoiding directly soiled with faeces. If the stall used when sampling bedding was soiled with faeces, the stall adjacent was used. Samples were placed in a 20 l pail, mixed thoroughly and two composite samples, each ~500 ml (two cups of volume), were labelled and stored. All composite samples were immediately placed into a cooler with ice packs and later transported to the University of Guelph, Kemptville Campus and stored at ~20°C until analysis.

**Processing and analysis of bedding samples**

Of the duplicate bedding samples, one set was dried at the University of Guelph, Kemptville Campus. Bedding samples were removed from the freezer and thawed, then oven-dried at 55°C for 48 h. The second set of samples was shipped frozen to the University of Prince Edward Island (Charlottetown, PEI, Canada) for bacterial culture of major pathogens (*Streptococcus* spp., all gram-negative bacteria *Klebsiella* spp., all gram-negative bacteria and *Klebsiella* spp.). Samples were sent for analysis on a weekly basis during the data collection period.

Bedding culture methods were based on procedures used by the University of Minnesota Laboratory of Udder Health, with modifications described below (Godden et al., 2008; Husfeldt et al., 2012). Culture media materials were based on those used by the Ohio State University, Ohio Agricultural Research and Development Center, with modifications described below (Hogan et al., 1990). Briefly, bedding samples were removed from the freezer and thawed overnight in the refrigerator prior to culture. A volume of 100 ml of each bedding sample was measured out in a sterile glass beaker. Sand has little air space between grains and was used as the guide for compacting other samples. Other bedding samples, like RMS and wood products, were lightly pressed into a beaker with sterile, gloved hands to reduce as much of the air space as possible. For longer bedding like straw, fibres were first cut (using sterilized scissors) into approximately 1.3 cm pieces so that they would lay flat in the beaker. The samples were then pressed in the same way as above. Bedding samples underwent a wash step by placing the sample in sterile filter bags and adding 30 ml of sterile water followed by mixing for 30 s, resting for 15 min and then mixing for another 30 s. After this wash step, a 100 ul sample of the liquid suspension was removed for the liquid compartment of the filter bag and used to make serial 10-fold dilutions from 1 : 102 to 1 : 104 in sterile distilled water. The diluted samples, 50 ul of sample, were then plated on media (described below) using a spiral plater (Auto plate 4000; Spiral Biotech, Norwood, MA, USA), as per the AOAC recognized method for analysing BCs in both liquid and solid foods (Standard Methods for the Examination of Dairy Products – 17th ed. 2004).

Three media were used to enumerate bacterial numbers: MacConkey agar, MAC (Alere, Ottawa, ON, Canada), MacConkey-inositol-carbenicillin agar, MAC IC (Alere) with 10 mg/l inositol (Sigma, Oakville, ON, Canada) and 75 mg/ml carbenicillin (Bioshop, Burlington, ON, Canada) and Edward’s modified medium agar, EMCO (WVR, Mississauga, ON, Canada) with 5 mg/l colistin sulphate (Sigma), 2.5 mg/l oxolinic acid (Sigma) and 50 ml/l of sheep blood (Alere). The MAC and MAC IC agars were incubated for 24 h at 37°C (Hogan et al., 1990). The EMCO agar was incubated for 48 h at 37°C (Sawant et al., 2002). Colony-forming units (cfu) per millilitre were determined for gram-negative bacteria (total growth on MAC), *Klebsiella* spp. (red-pink colonies on MAC IC) and *Streptococci* and *Streptococci* like organisms (total growth on EMCO) as per the spiral plater grid chart (Auto plate 4000; Spiral Biotech). Bacterial count was expressed as cfu/ml, as reported by Godden et al. (2008), Harrison et al. (2008) and Husfeldt et al. (2012).

**Milk quality data**

Data on milk quality (farm average SCC) and cow demographics (lactation, DIM) were retrieved from CanWest DHI (Guelph, ON, Canada) reports using the test closest to the first visit and another test closest to the last visit for all cows in each farm. Farm average SCC (average SCC for the farm, weighted by each cow’s milk production) from the two DHI tests was then used for analysis to assess milk quality at the farm level. Bulk milk SCC (*BMSCC*) and BC (*BMBC*) using Bactoscan test (results in individual bacteria count (IBC/ml)) were also obtained, for a week prior to each farm visit, from the Dairy Farmers of Ontario (Mississauga, ON, Canada).

**Statistical analyses**

For hygiene scoring, the observers undertook prior training to ensure consistent methodology. In brief, observers were trained first using images of body parts to assess hygiene, then observers assessed together a subset of animals and reinforced scoring uniformity between observers. Lastly, inter-observer reliability was then evaluated by comparing hygiene scores from a farm assessed independently by the two observers. Cohen’s Kappa values were: lower leg = 0.81, udder = 0.82 and upper leg/flank = 0.79.

Bedding substrates were classified by farm: new sand (*n* = 12), straw (straw and hay; *n* = 33), wood products (shavings and sawdust; *n* = 17) and RMSs-compost and digestate (*n* = 8). In total data from 70 dairy farms were analysed. From 75 farms visited, those with bedding packs were
excluded from analysis due to small sample (n = 2), and 3 other farms were removed because they were the only farms using a specific bedding type (straw with lime and water, peat moss and organic compost, respectively). Further exclusion of farms from specific analyses was performed if information for a specific outcome variable was not available (see Table 1). Data were summarized per farm per visit for the statistical analyses. Descriptive statistical analysis was performed using the UNIVARIATE procedure of SAS (2013, version 9.3; SAS Institute Inc., Cary, NC, USA).

Prior to analyses, all data were screened for normality by assessing the distribution of data with the Anderson–Darling test and any outliers using the UNIVARIATE procedure. In addition, the UNIVARIATE procedure was also used to check the residuals for normality using the Shapiro–Wilk test. Linearity was assessed when plotting residuals, and normality of residuals was assessed using a probability plot. Mean herd SCC, BMSCC, BMBC and all bacterial culture count were right skewed and were, therefore, transformed by taking their natural logarithm; this transformation normalized these data.

All individual predictor variables (see Supplementary Material S1) that were considered for inclusion in the multivariable models were tested in univariable models using the MIXED procedure of SAS. Covariance structures were selected on the basis of best fit according to Schwarz’s Bayesian information criterion. Compound symmetry covariance structure was selected for all outcome variables, except for all gram-negative bacteria as an outcome variable, in which the autoregressive (1) structure was selected. Predictor variables with P < 0.25 in the univariable analysis were included in the multivariable models (Dohoo et al., 2009).

Pearson correlation coefficients were calculated for all predictor variables that were considered for inclusion in the multivariable model to detect issues of collinearity. Consequently, if the correlation coefficient was greater than [0.8], then either the variable that made the most biological sense was used or that with the lower P-value. In all multivariable models, manual backward elimination was used to remove any variables with P > 0.10; only those variables retained were considered that were significant at P ≤ 0.05 and tendencies were considered at 0.05 < P ≤ 0.10. If the P-value of an interaction term was <0.05, it was considered, otherwise interaction terms were disregarded. A confounding variable was defined as a non-intervening variable whose removal resulted in a >25% change in the coefficients of significant variables in the final model.

If housing type was associated with an outcome variable in the final multivariable mixed model, additional housing-specific aspects were investigated in a separate multivariable analysis, by housing type, using the procedure described above. Housing-specific aspects for free-stall barns included: surface of the alleyways (rubber, concrete), frequency of

<table>
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<th>Variable</th>
<th>Farms1</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
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<td>70</td>
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<td>Milk yield (kg/day)</td>
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<td>–</td>
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<tr>
<td>Bulk milk SCC (×1000 cells/ml)4</td>
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<td>Bulk milk bacteria count (×1000 IBC/ml)5</td>
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<td>18</td>
<td>–</td>
<td>3</td>
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<td>Relative humidity daily average (%)8</td>
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<td>92.0</td>
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</table>

SCC = somatic cell count; IBC = individual bacteria count; DHI = dairy herd improvement.

1Number of farms with data available for each measure.
2Data from cows at the beginning of the study period.
3SCC = mean data from two DHI tests: one closest to the first visit and one closest to the last visit.
4Data from a week prior to each of the three farm visits, 7 days apart.
5BMBC = bulk milk bacteria count (IBC/ml) assessed 2x/week for a week prior to each of the three farm visits, 7 days apart.
6Dimensions were measured by research personnel during one of the visits.
7Management practices described by producers.
8Average temperature from the closest Environment Canada weather station in 7 days before each visit.

*Data presented for farms that had DHI recordings 10 to 12 tests/year.
alleyways cleaning in a day and neck rail distance from rear curb. Housing-specific aspects for tie-stall barns included: herd access to pasture (yes/no), manger height (cm) and trainer height (cm).

The Kenward–Rogers adjustment for df was included in each model. Least squares means and standard errors were generated for all the models using the LSMEANS statement in the MIXED procedure. Least squares means comparisons were separated with PDIFF statement for all the models constructed. The Tukey–Kramer adjustment was used to compare the least squares means for bedding types. Visit was included as a repeated measure for all models constructed and, as such, included as one of the predictor variables (except for the outcome variable farm weighted average SCC from DHI). Farm was included as a random effect for all models constructed. For each model, validation was performed according to Dohoo et al. (2009). The normality of the residuals was assessed by observing the normal probability plots of the residuals and standardized residuals (Q–Q plot), as well as by looking at the plot of the standardized residuals against the fitted values (see Supplementary Material S2 for plots of model fit for one of each constructed model described below). Influential points and outliers were identified by looking at the Cook’s distance values, leverage and difference in fit (DIFFITS) values.

Four separate multivariable linear mixed models were constructed to investigate associations of outcome variables % DM and bacterial content (separate for *Streptococcus* spp., all gram-negatives and *Klebsiella* spp.) with the predictor variable unused bedding types (straw, sawdust, wood products and RMS). Each one of those models was adjusted for the variables temperature, relative humidity of the environment and visit (see Supplementary Material S3 for example of SAS coding).

Four multivariable linear mixed models were constructed to investigate the associations of the outcome variables % DM and bacterial content (separate for *Streptococcus* spp., all gram-negatives and *Klebsiella* spp.) with the predictor variable used bedding type (straw, sawdust, wood products and RMS). For the analysis of used bedding % DM, the predictor variables included in the multivariable model were used bedding type, base of the stall and humidity of the environment. For the analysis of *Streptococcus* spp. in used bedding, the predictor variables included in the multivariable model were used bedding type, % DM in used bedding, days since additional bedding was added to the stall, base of the stall and visit. For the analysis of all gram-negatives in used bedding, the predictor variables included in the multivariable model were bedding type, base of the stall, weighed stocking density (number of cows/number of stalls for each pen, averaged across all milking cows pens) and days since additional bedding was added to the stall. For the analysis of *Klebsiella* spp. in used bedding the predictor variables included in the multivariable model were bedding type, housing and how often stalls were scraped (number per day).

All three body hygiene scores (lower legs, udder, upper legs and flanks) were analysed in separate multivariable analyses, by housing type, since housing type was associated ($P < 0.001$) with both the percentage of cows with dirty lower legs and with dirty upper legs and flanks ($P < 0.001$).

When tested for collinearity, predictor variables for hygiene of udder and upper legs and flanks in free-stall barns that were correlated were: how often stalls were scraped and the number of cows milking ($r = 0.82$), and DIM and neck rail height ($r = 0.84$). Variables kept for the multivariable model were how often stalls were scraped and DIM.

Three multivariable linear mixed models were constructed to investigate the associations of various predictors with the outcome hygiene – percentage of dirty cows per farm (body areas: lower legs, udder, upper legs and flank) in free-stall barns. For the analysis of percentage of cows with dirty lower legs, predictor variables were used bedding type, weighed stock density, temperature, milk system, how often stalls were scraped, number of times cows were milked and stall length. For the analysis of percentage of cows with dirty udders, predictor variables were frequency of alley cleaning per day, milking system, neck rail distance from rear curb, neck rail height (cm), number of cows milking, DIM and milk yield. For the analysis of percentage of cows with dirty upper legs, flank predictor variables were bedding type, days since additional bedding was added to the stall, frequency of alley cleaning per day, weighed stock density, milking system, base of the stall, how often stalls were scraped, neck rail height, surface of the alley ways, number of cows milking, DIM and milk yield.

Three multivariable linear mixed models were constructed to investigate associations of various predictors with the outcome hygiene – percentage of dirty cows per farm (body areas: lower legs, udder, upper legs and flank) in tie-stall barns. For the analysis of percentage of cows with dirty lower legs, predictor variables were used bedding type, herd access to pasture, how often stalls were scraped (number per day), manger height (cm) and trainer height (cm), lime. For the analysis of percentage of cows with dirty udders, predictor variables were bedding type, days since additional bedding was added and % DM in bedding.

A multivariable linear mixed model was constructed to investigate the associations of the outcome variable farm weighted average SCC from DHI with predictor variables: % DM of used bedding type, how often stalls were scraped (number per day), stall width, neck rail distance from rear curb, DIM, milk yield and milking system.

A multivariable linear mixed model was constructed to investigate the associations of the outcome variable bulk milk quality (BMSCC) with predictor variables: bacterial content in used bedding (*Streptococcus* spp., all gram-negatives), how often stalls were scraped (number per day), stall width, base of the stall, lime and use of gloves. A positive significant interaction ($P < 0.01$) was detected between the variables stall width and distance (length) from the back of the stall to the neck rail for BMBC. The association of these variables was plotted to further assess and verify that the relationship was linear (Supplementary Material Figure S1).
A multivariable linear mixed model was constructed to investigate the associations of the outcome variable bulk milk quality BMBC with predictor variables: stall width and neck rail distance from rear curb.

Results

Inter-observer reliability
High levels of agreement were found between the observers when scores, assessed independently, were categorized as good or poor hygiene (Cohen’s Kappa values for lower leg \(= 1.00\); 95% CI = 1.00 to 1.00, udder = 0.79; 95% CI = 0.59 to 0.98, upper leg/ flank = 1.00; 95% CI = 1.00 to 1.00).

Characteristics of surveyed farms
In total, data from 70 dairy farms were analysed. Data on cow demographics, milk yield and quality, and stall size and maintenance are summarized in Table 1. Lactating cows were housed in free-stall (\(n = 44\)) and tie-stall (\(n = 26\)) barns. The frequency of alleyway cleaning in free-stall barns was 12.4 ± 11.0 times/day; mean ± SD. At 24 (55%) of 44 free-stall barns, the milking system used was an automatic milking system (AMS), while the 20 (45%) other farms used a milking parlour. At 30 of the free-stall barns, mattresses were used as a stall surface whereas 14 barns had deep bedding. At 11 (42%) of the 26 tie-stall barns, cows had access to pasture in the summer and during data collection period (fall/winter), the remaining 15 farms stayed in the tie-stall year-round. At 3 (7%) of the 44 free-stall barns, cows had access to pasture in the summer, the remaining 41 farms stayed in the free-stall year-round. Producers added lime to the bedding after placing on the stall in 12 (46%) of the 26 tie-stall barns. Producers added lime to the bedding after placing on the stall in 21% of the 44 free-stall barns.

Cow and milking information
Average size of the 70 farms was 100 lactating cows, ranging from 25 to 404, and mean DIM of 170 (Table 1). Average milk production was 32.7 kg of milk/day. Producers used one of three milk systems: AMS (\(n = 25\); 36%), parlour (\(n = 21\); 30%) or milked in the tie-stall (\(n = 24\); 34%). On farms with a milking parlour or tie-stall, cows were milked two (\(n = 37\); 82%) or three (\(n = 8\); 18%) times/day. Cows in AMS farms were milked on average 2.6 ± 0.35 times/day. When milking cows in the farms, all employees used gloves (\(n = 28\); 62%), some employees used gloves (\(n = 11\); 24%) and none of the employees used gloves (\(n = 13\)). After milking cows, post dip was applied in either spray form (\(n = 24\); 34%) or dipping form (\(n = 46\); 66%) as one producer in AMS manually dipped all cows twice/day (AM, PM).

Hygiene score
All three body hygiene scores (lower legs, udder, upper legs and flanks) were analysed in a separate multivariable analysis by housing, since housing type was associated (\(P < 0.001\)) with both the percentage of cows with dirty lower legs and with dirty upper legs and flanks (\(P < 0.001\)). Cows in free-stall barns more often had dirty lower legs, upper legs and flanks compared to tie-stall barns (\(P < 0.001\); Table 2).

In the 24 free-stall AMS barns, there was an 11.4% point increase in the number of cows that had dirty lower legs compared to cows of farms with a milking parlour (\(n = 20\); Table 3). In free-stall barns, proportion of cows with dirty udders, upper legs and flank increased with a decreased frequency of alleyways cleaning (12.4 ± 11 cleanings/day; mean ± SD; Table 3). Proportion of cows with dirty udders and upper legs and flanks was higher in farms with a higher average DIM. Farms with mattresses tended to have more cows with dirty upper legs and flank compared to farms with deep bedding (Table 3). Proportion of cows with poor udder hygiene (percentage of cows per farm with a score of ≥3 for udder hygiene) among farms varied per visit (\(P = 0.04\); Table 3), being higher at the third visit (Table 3).

For farms with a tie-stall barn with cow access to pasture, there was a 17.7% point increase in the number of cows with dirty legs compared to farms without access to pasture (Table 4). Farms that utilized lime in their bedding tended to have a 16.2% point increase in the number of cows with dirty legs. Farms that added additional bedding every other day tended to have a 7.0% point increase in the number of cows with dirty udders compared to those that added bedding every day.

Table 2: Proportion of cows with poor hygiene scores in 70 Ontario, Canada, dairy cow farms, by housing type and body area

<table>
<thead>
<tr>
<th>Area</th>
<th>Tie-stall1</th>
<th>Free-stall2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SD</td>
<td>Minimum Maximum</td>
</tr>
<tr>
<td>Upper leg – flank</td>
<td>40±</td>
<td>2.5</td>
</tr>
<tr>
<td>Lower legs</td>
<td>26±</td>
<td>0.0</td>
</tr>
<tr>
<td>Udder</td>
<td>31±</td>
<td>0.0</td>
</tr>
</tbody>
</table>

1Means of results from three visits, within a row with different superscripts differ (\(P < 0.01\)).
2Hygiene score was assessed on a 4-point scale (1 = very clean to 4 = very dirty) during each of the 3 visits, 7 day apart. Hygiene scores ≥3 were classified as poor.
3Tie-stall barns (\(n = 26\)), a total of 986 cows scored.
4Free-stall barns (\(n = 44\)), a total of 1784 cows scored.
**Table 3**  Final mixed model for factors associated with proportion of dairy cows with poor hygiene of the upper legs-flank, udder and lower legs in free-stall barns

<table>
<thead>
<tr>
<th>Variable</th>
<th>Upper legs – flank</th>
<th>Udder</th>
<th>Lower legs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>$-16.5$</td>
<td>$-26.4$</td>
<td>$81.6$</td>
</tr>
<tr>
<td>Milking system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Automated milking system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milking parlour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alleyways cleaning frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days in milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stall base</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deep bedding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mattress</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Analysed model included data from 44 farms, each with 3 observations (proportion of cows with poor hygiene) per farm.
2Hygiene score was assessed on a 4-point scale (1 = very clean to 4 = very dirty) and classified as poor if ≥3. Proportion of cows with poor hygiene per farm was calculated during each of the three visits, 7 day apart.
3Free-stall barns (n = 44), n = 1784 cows scored.
4$\beta$ = estimated regression coefficient.
5Ref = Reference category.
6Management practices described by producers.
7Visit was considered a repeated effect. Covariance parameter estimates for the herd, visit and residual were, respectively, for lower legs $0.03$; for every 10-day increase in DIM, average weighted farm SCC of the 64 farms participating in DHI was 184 000 cells/ml (median: 161 000 cells/ml), whereas BMSCC (n = 70) averaged 186 000 cells/ml (median: 180 000 cells/ml), and BMBC averaged 18 000 IBC/ml (median: 10 000 IBC/ml) (Table 1). Mean SCC was higher in farms with higher average DIM (intercept $= 12.5; \beta = 0.005; SE = 0.002; P = 0.03$); for every 10-day increase in DIM, average weighted farm SCC increased by 10 000 cells/ml. Stall width tended to be associated with higher SCC (intercept $= 10.0; \beta = 0.15; SE = 0.11; P = 0.07$) for every 5-cm increase in stall width, average SCC tended to decrease by 5000 cells/ml. A weak positive correlation ($r = 0.23; P < 0.001$) was reported for stall width and frequency/day of scraping stalls. However, stall length and frequency of scraping stalls were not correlated ($r = -0.11; P = 0.27$).

Bulk milk bacteria count was higher for farms using RMS bedding, followed by wood products, straw and sand (Table 5). Wider stalls were associated with lower BMBC; for every 5 cm increase in stall width, average BMBC decreased by 5000 IBC/ml. Lower % DM of used bedding was associated with higher BMBC; for every 10% decrease in used bedding % DM, average BMBC increased by 10 000 IBC/ml. An interaction ($P < 0.01$) was observed between stall width and distance (length) from the back of the stall to the neck rail for BMBC, indicating that BMBC was lower on farms with both longer and wider stalls.

### Milk quality

Weighted average farm SCC of the 64 farms participating in DHI was 184 000 cells/ml (median: 161 000 cells/ml), whereas BMSCC (n = 70) averaged 186 000 cells/ml (median: 180 000 cells/ml), and BMBC averaged 18 000 IBC/ml (median: 10 000 IBC/ml) (Table 1). Mean SCC was higher in farms with higher average DIM (intercept $= 12.5; \beta = 0.005; SE = 0.002; P = 0.03$); for every 10-day increase in DIM, average weighted farm SCC increased by 10 000 cells/ml. Stall width tended to be associated with higher SCC (intercept $= 10.0; \beta = 0.15; SE = 0.11; P = 0.07$) for every 5-cm increase in stall width, average SCC tended to decrease by 5000 cells/ml. A weak positive correlation ($r = 0.23; P < 0.001$) was reported for stall width and frequency/day of scraping stalls. However, stall length and frequency of scraping stalls were not correlated ($r = -0.11; P = 0.27$).

Bulk milk bacteria count was higher for farms using RMS bedding, followed by wood products, straw and sand (Table 5). Wider stalls were associated with lower BMBC; for every 5 cm increase in stall width, average BMBC decreased by 5000 IBC/ml. Lower % DM of used bedding was associated with higher BMBC; for every 10% decrease in used bedding % DM, average BMBC increased by 10 000 IBC/ml. An interaction ($P < 0.01$) was observed between stall width and distance (length) from the back of the stall to the neck rail for BMBC, indicating that BMBC was lower on farms with both longer and wider stalls.

### Bedding types and managements

Organic bedding was used most commonly (71% of farms), followed by inorganic bedding (17%) and RMS (11%). Surface of the milking cow stalls consisted either of mattresses (n = 56) or deep bedding (n = 14). On average, farms added additional bedding to stalls every 3.0 ± 5.0 day (range: 0 to 28 day) (Table 1). Frequency of stall cleaning varied greatly among farms ranging from 1 to 8 cleanings per day (Table 1).

### Bedding samples

Unused RMS bedding types had lower % DM and higher Streptococcus spp. counts compared to all other bedding types (Table 6). Unused straw bedding was higher than all other bedding types in gram-negative and Klebsiella spp. counts. In used bedding, sand was driest, compared to straw and wood products, and RMS types (Table 6). Higher relative humidity of the environment tended to be associated with lower % DM (intercept $= 87.7; \beta = -0.162; SE = 0.09; P = 0.07$). Higher % DM in used bedding was associated with lower Streptococcus spp. count (intercept $= 17.58; \beta = -0.03; SE = 0.01; P < 0.01$). Streptococcus spp. and all gram-negative counts increased with increasing days since additional bedding was added (intercept $= 17.58; \beta = 0.11; SE = 0.01; P < 0.001$ and intercept $= 9.85; \beta = 0.15; SE = 0.01; P < 0.001$, respectively). In used bedding, gram-negative counts were higher in RMS compared to straw and sand, and compared to wood products.
the farms presented in this study were within the spectrum travelling factors and time availability, demographics of given that a random sample was not feasible considering While the study farm population was a convenience sample, Discussion

Table 4 Final mixed model\(^1\) for factors associated with proportion of dairy cows with poor hygiene of the upper legs-flank, udder and lower legs\(^2\) in tie-stall barn\(^3\) during the period from October 2014 to February 2015

<table>
<thead>
<tr>
<th>Variable</th>
<th>Upper legs – flank</th>
<th>Udder</th>
<th>Lower legs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-</td>
<td>29.1</td>
<td>45.8</td>
</tr>
<tr>
<td>Access to pasture</td>
<td>-</td>
<td>3.93</td>
<td>8.95</td>
</tr>
<tr>
<td>No</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yes</td>
<td>-</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Product added to bedding</td>
<td>-</td>
<td>-17.7</td>
<td>8.31</td>
</tr>
<tr>
<td>No</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>-</td>
<td>-16.2</td>
<td>8.41</td>
</tr>
<tr>
<td>Days since additional bedding was added (number/day)(^5)</td>
<td>-</td>
<td>7.0</td>
<td>4.36</td>
</tr>
</tbody>
</table>

\(^1\)Analysed model included data from 26 farms, each with 3 observations (proportion of cows with poor hygiene) per farm.  
\(^2\)Hygiene score was assessed on a 4-point scale (1 = very clean to 4 = very dirty) and classified as poor if \( \geq 3 \). Proportion of cows with poor hygiene per farm was calculated during each of the three visits, 7 day apart. Visit was considered a repeated effect. Covariance parameter estimates for the herd, visit and residual were, respectively, for udder = 358, -24 and 74; and lower legs = 318, 0 and 121.  
\(^3\)Tie-stall barns (\( n = 26 \)), a total of 986 cows scored.  
\(^4\)\( \beta \) = estimated regression coefficient.  
\(^5\)Ref = Reference category.  
\(^6\)Management practices described by producer.

Table 5 Bulk milk bacterial\(^1\)-\(^2\) count of the 70 participating dairy cow farms in Ontario, Canada during the period from October 2014 to February 2015

<table>
<thead>
<tr>
<th>Bedding type</th>
<th>Bacterial count (ln IBC/ml)(^3)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>9.0(^4)(^5)</td>
<td>0.16</td>
</tr>
<tr>
<td>Straw</td>
<td>9.4(^4)(^5)</td>
<td>0.10</td>
</tr>
<tr>
<td>Wood products</td>
<td>9.5(^4)(^5)</td>
<td>0.13</td>
</tr>
<tr>
<td>Recycled manure solids</td>
<td>9.9(^6)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

\(^1\)Analysed with a multivariable mixed model that included data from 70 farms, each with 3 observations (average of 2 samples during week prior to each farm visit) per farm. Model accounted for stall width and neck rail distance from back of the stall in milking cow stalls per farm.  
\(^2\)Dairy Farmers of Ontario bulk milk bacteria count \( \times \) week for a week prior to each of the three farm visits, 7 day apart. Visit was considered a repeated effect. Covariance parameter estimates for the herd, visit and residual were 0.1, 0.01 and 0.3, respectively.  
\(^3\)Bacterial count was analysed using natural logarithm (ln) values of Bactoscan test results of individual bacterial count (IBC/ml).  
\(^4\)Tendency for difference between sand and straw (\( P = 0.08 \)).  
\(^5\)Tendency for difference between straw and wood (\( P = 0.06 \)).

Discussion

While the study farm population was a convenience sample, given that a random sample was not feasible considering travelling factors and time availability, demographics of the farms presented in this study were within the spectrum of farms in this region (Canadian Dairy Information Centre, 2018). Further, housing and milking management practices on the study farms were similar to those farms studied in the United States (Rowbotham and Ruegg, 2015) and United Kingdom (Bradley et al., 2018). Tie-stall barns comprise 68% of the total barns in Ontario (2262 dairy farms), while free-stall barns comprise 32% of the farms. When compared, the distribution of housing systems studied in this research deviates from that of Ontario’s (Canadian Dairy Information Centre, 2018), perhaps because researchers aimed to study different types of bedding used in dairy farms as part of the convenience sample rather than an explicit focus on housing system distribution. The average size of a herd enrolled in a milk (Canadian Dairy Information Centre, 2018) recording program in Ontario (92.6 cows) and in Canada (96.5 cows) is close to the average herd size in this study, in which all farms had to be enrolled in a milk recording program (Canadian Dairy Information Centre, 2018). For robotic milking systems, 98.9% of the barns that have robotic milking systems are free-stall barns (Canadian Dairy Information Centre, 2018), while in this study all the farms that had robotic milking system were free-stall barns. The mean number of cows in Ontario housed tie-stall barns and free-stall barns falls within the minimum and maximum number of cows milking farm types in this study. Cows enrolled in official milk recording system in Canada produce on average 10 528 kg of milk per lactation (based on 305 day), which is very similar to the reported production of the cows in the herds included in this study and a study in larger hers in the United States (Rowbotham and Ruegg, 2015). One of the gaps in the literature that this study aimed to fill was compared different bedding types used for dairy cows as bedding in Canada, including straw. Although there is no specific report of bedding-type usage in dairy

(Table 6). Klebsiella spp. counts in used bedding were lower in wood products than all other bedding types sampled (Table 6).
Bacterial count in different bedding types

Table 6 | Results of multivariable mixed model for dry matter and bacterial counts for unused and used bedding types for dairy cow farms (n = 70) visited three times, 7 days apart during the period from October 2014 to February 2015.1,2

<table>
<thead>
<tr>
<th>Content</th>
<th>Sand</th>
<th>Straw</th>
<th>Wood products</th>
<th>RMS3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unused bedding</strong></td>
<td><strong>% DM</strong></td>
<td><strong>Bacterial counts (ln cfu/ml)</strong></td>
<td><strong>Bacterial counts (ln cfu/ml)</strong></td>
<td><strong>Bacterial counts (ln cfu/ml)</strong></td>
</tr>
<tr>
<td></td>
<td>92.1 ± 1.75</td>
<td>88.3 ± 0.76</td>
<td>85.3 ± 1.09</td>
<td>37.9 ± 1.50</td>
</tr>
<tr>
<td></td>
<td>2.5 ± 0.96</td>
<td>2.5 ± 0.57</td>
<td>2.6 ± 1.04</td>
<td>4.3 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>13.5 ± 0.64</td>
<td>13.8 ± 0.36</td>
<td>10.8 ± 0.95</td>
<td>11.7 ± 0.57</td>
</tr>
<tr>
<td><strong>RMS</strong></td>
<td>2.6 ± 0.62</td>
<td>4.3 ± 0.89</td>
<td>5.9 ± 0.79</td>
<td>10.9 ± 1.16</td>
</tr>
</tbody>
</table>

1,2,3 Mean within the row with different superscripts differ (P < 0.01).

1Analysed model included data from 70 farms, each with 3 observations (samples) per farm.

2Visit was considered a repeated effect. Covariance parameter estimates for the herd, visit and residual were, respectively, for unused bedding % DM = 11, 1 and 10; unused bedding bacterial counts (Streptococcus spp. = 6, 0.1 and 12; all gram-negative = 4, 0.1 and 14; Klebsiella spp. = 7, −0.2, and 13); used bedding % DM = 22.0, 0.5 and 30; used bedding bacterial counts (Streptococcus spp. = 1.2, 0.1 and 1.5; all gram-negative = 0.5, 0.3 and 12; Klebsiella spp. = 5, −0.01 and 17).

3RMS = Recycled manure solids from composted manure or digestate.

4Adjusted for temperature, humidity of the environment and visit.

5Tendency for difference between straw and wood (P = 0.07).

6Bacterial counts were analysed after natural logarithm transformation; ln colony forming units (cfu/ml) are reported for presentation purposes.

7Adjusted for humidity of the environment.

8Adjusted for days since additional bedding was added and visit.

farms in Canada, there have been larger survey studies that report the use of straw in a vast majority of the farms surveyed (92% of 100 tie-stall herds; Nash et al., 2016) and a commonly used bedding type in another larger study (20% of 87 free-stall herds; Zaffino Heyerhoff et al., 2014). Management of stalls in the present study is consistent with those reported by Bradley et al. (2018); those researchers completed a study in the UK on 125 farms and reported a summary of bedding management with most farms scraping manure from stalls 2 to 3 times a day, more (63 farms) using mattresses with a covering bedding material compared to deep bedding (48 farms) and with bedding classifications including sawdust, RMS and sand. For milking practices, the same study reported 2 milkings per day for most farms (83 farms), as in the present study. Unsurprisingly, post dipping, as done on our study herds, is a common practice in North America (Rowbotham and Ruegg, 2015).

In the present study, bedding types were classified as sand, wood products, straw and RMS. As expected, unused sand (inorganic bedding) was very dry (92% DM), which also likely explained the drier used sand bedding samples; good drainage properties of sand help maintain this high % DM. Dry matter percentage of used sand was high in this study; other researchers (Zdanowicz et al., 2004; 95% DM; Kristula et al., 2005; 95% DM) have also reported sand as a very dry bedding. Likewise, the results for RMS DM content are in agreement with other research that reported this material to be quite wet (~40% DM or lower), but also that RMS dries, albeit only slightly, over time once placed into the stalls (Husfeldt et al., 2012; Cole and Hogan, 2016). In the current study, % DM of straw and wood products were relatively close in both unused and used bedding. Used straw and wood bedding material had lower % DM compared to the unused material, whereas used RMS bedding % DM was higher than unused RMS and sand % DM did not differ between unused and used bedding. Zdanowicz et al. (2004) reported that % DM in used sawdust bedding decreased over time after bedding was added to the stall, but % DM of sand remained very high between sampling days. An increase in % DM content once RMS was used as stall bedding has been reported by others (Husfeldt et al., 2012; Cole and Hogan, 2016).

As can be expected, relative humidity of the environment and % DM in used bedding tended to be negatively associated. Lower % DM in used bedding was also associated with a higher Streptococcus spp. count. These results are in agreement with Godden et al. (2008) who noted that bacterial growth can be influenced by moisture/humidity. Both Hogan et al. (1990) and Zehner et al. (1986) have reported a positive association between relative humidity of the environment and BC results in used bedding. Zdanowicz et al. (2004) also reported higher BC (coliforms, Klebsiella spp. and Streptococcus spp.) in bedding samples of used sawdust with lower % DM. Interestingly, for used sand bedding, Zdanowicz et al. (2004) reported a positive association between % DM and coliform counts. Unfortunately, these researchers did not offer any explanation for this result.
and, thus, should be viewed with caution, particularly since their finding contradicts our own and others who have reported that BC is typically lower in drier samples.

Bedding samples were sent for analysis on a weekly basis, well within an acceptable timeframe, given the results of Homerosky and Hogan (2015), to not significantly affect the counts of gram-negative, coliform, *Klebsiella* spp. or Streptococci bacteria in frozen samples of fresh sand, sawdust or RMSs bedding. Nevertheless, those authors reported difference in BCs with reduced BC recovery on frozen samples compared to fresh samples. However, given that all samples were handled in the same manner, the freezing effect would not have an impact in the comparisons reported in this study, instead it could have potentially reduced total counts of bacteria in all bedding types.

Several researchers have demonstrated that BC varies among bedding types, with inorganic bedding containing (Zdanowicz et al., 2004; Bradley et al., 2018), or promoting growth (Godden et al., 2008) of, fewer bacteria than organic bedding types. Zehner et al. (1986) tested the ability of different sterilized bedding types to promote growth of environmental bacteria (*E. coli*, *Klebsiella pneumoniae* and *S. iberis*) under controlled conditions and reported that growth of all bacteria was highest in recycled dried manure, second highest in straw, third in hardwood chips and lowest in paper and sawdust. Our results indicate that *Streptococcus* spp. count in unused bedding was higher in RMS when compared to sand, straw and wood products, whereas gram-negative bacteria counts were highest in straw, compared to RMS, and compared to sand and wood. *Klebsiella* spp. BC was higher in straw than all the other bedding types sampled. Similarly, Godden et al. (2008) found that growth support of *K. pneumoniae* was similar among clean sand, recycled sand, digested manure solids and shavings bedding; these researchers did not report the bacterial growth support of straw bedding.

For used bedding samples, our findings are comparable to previous studies (e.g. Zdanowicz et al., 2004). For example, in the current study, gram-negative count in used bedding was high in RMS, compared to straw and sand, and compared to wood products. Rowbotham and Ruegg (2016a) also reported that total gram-negative bacteria counts were 100 to 1000 times lower in used sand bedding v. used RMS bedding. It is noteworthy that wood-based bedding contained the lowest gram-negative bacteria, Zehner et al. (1986) reported low for sterilized unused bedding samples. Comparably, counts of *Klebsiella* spp. in used bedding were similar for sand, straw and RMS, while lower in wood products. Contrary to our results, Zdanowicz et al. (2004) reported that sawdust had higher coliform and *Klebsiella* spp. counts compared with sand; these results were positively correlated with BC on teat ends. The disagreement in results could be due to the grouping of wood types bedding (sawdust, shavings and wood chips) in our study v. just sawdust used in their study. For example, in an experimental study by Zehner et al. (1986), fine hardwood chips resulted in more *Klebsiella* spp. growth than did softwood sawdust. Furthermore, *Klebsiella* spp. growth was much higher in both recycled manure and straw than in softwood sawdust (Zehner et al., 1986). Similar to Rowbotham and Ruegg (2016b), we detected high levels of *Klebsiella* spp. in used RMS. However, when compared to other bedding types, only RMS differed from wood products, whereas Rowbotham and Ruegg (2016b) detected a difference between sand and RMS. These differences between studies may be due to differences in study design. In their experimental study, Rowbotham and Ruegg (2016b) added new bedding twice weekly, whereas in our observational study, sand bedding was added primarily on either a weekly or biweekly basis, depending on the participating farms. The longer time interval in our study may have promoted the growth of bacteria on sand, which may have prevented any observable differences in the current study between bedding types (with the exception of the RMS). Additionally, the longer time interval in our study may have increased the proportion of faecal matter in the bedding and thus, also increasing the BCs in the bedding. In a study in which stall cleanliness and BCs in used bedding types (sand, sawdust) were examined, it was reported that dirtier stalls (i.e. greater number of squares containing any visible faecal matter) contained higher bacteria counts (Coliforms – lactose positive colonies on MC agar, Streptococci and *Klebsiella* spp.) (Zdanowicz et al., 2004). However, stall cleanliness was not assessed in the current study, and thus, although plausible, BC increase with increased stall dirtiness cannot be confirmed.

*Streptococcus* spp. were high in all used bedding types, but no differences between bedding types were present. This is consistent with previous work showing environmental bacteria numbers are high for all bedding types typically used for cows, including sand and RMS, faeces and feed (Hogan and Smith, 2012; Rowbotham and Ruegg, 2016b). In a recent study, Bradley et al. (2018) reported that *Streptococcus/ Enterococcus* spp. counts were lower in sand when compared with RMS and sawdust, but no effect was noted between RMS and sawdust used bedding. Those same authors highlighted a large variation in BC within the bedding types studied; thus, that variation within bedding groups could have influenced their results.

Management plays a key role in reducing environmental pathogen exposure to dairy cows. The number of days since each bedding substrate is placed upon the lying surface can affect bacterial growth (Zdanowicz et al., 2004; Kristula et al., 2005; Cole and Hogan, 2016). For instance, in the present study, *Streptococcus* spp. and gram-negative BC increased with days since additional bedding was added. Further, low % DM of used bedding was associated with higher BMBC. It is possible that higher BC in low % DM bedding resulted in higher transfer of bacteria from the bedding to cow teat ends. Evidence has been reported of a strong association between bacteria in bedding and on cow teats and higher bacteria counts for bedding samples with low % DM (Zdanowicz et al., 2004; Rowbotham and Ruegg, 2016b). Collectively, other research and the results of our analysis for bedding addition support the recommendation
that bedding be added and replaced frequently to reduce exposure to environmental mastitis pathogens. Farms with RMS bedding had higher BMBC compared to farms with any of the other bedding types. Bradley et al. (2018) reported high BCs in RMS, those authors reported no association between BCs in bulk milk with farms using RMS bedding as compared to sawdust and sand. Rowbotham and Ruegg (2015) reported no association between total BC in milk and bedding type. However, it has been argued that ‘absence of evidence is not evidence of absence’ (Altman and Bland, 1995) and it is often unclear which statistical tests were used to claim equivalence (e.g. Bello and Renter, 2018), as such testing was not reported. Conversely, our study reported a marginal difference between BMSCC and bedding type, whereas Rowbotham and Ruegg (2015) reported that farms using inorganic bedding had lower BMSCC. The differences between these two studies may due to study design. Our study was a cross-sectional study, while Rowbotham and Ruegg (2015) was a longitudinal study, with farms followed over a 2-year period. Given that SCC (in response to environmental pathogens) can elevate for short period of time (De Haas et al., 2004), the longitudinal observation period would have captured more of those potential elevations, whereas our own study design would not. Another possible explanation could be that a smaller sample size of farms, given the inherent large variability in BMSCC among farms, would limit our results in this area. Another plausible explanation for different results between Bradley et al. (2018) and the current study results may be due to variation in bedding management practices. As noted in this study, BC in bedding changes with days since additional bedding was added. Thus, the variation in bedding replenishment between farms used in the study of Bradley et al. (2018) and the present study could have accounted for the lack of influence of BC in the bulk milk for different bedding types in their study. For instance, some farms used in the mentioned study replenished bedding as often as twice daily. Additionally, the study by Bradley et al. (2018) was carried out during winter and spring, thus result interpretations might not extrapolate to other seasons, such as summer. The same limitations apply to this study, as data collection was completed during the fall/winter season. Currently, there is no standardized method of evaluating bacterial content in bedding. The methodology used in this study for evaluating bacterial content has been challenged and argued that it is not valid in terms of predicting risk of bacterial transfer from bedding into bulk milk (Bradley et al., 2018). However, Bradley et al. (2018) did not provide any evidence that their preferred method was indeed a valid method. Further, with respect to volume v. weight in collection of bedding samples, given the very different densities of the various bedding materials analysed in the present study, measuring samples based on volume instead of weight was used, as it is the preferred method when comparing different bedding materials with different densities (Harrison et al., 2008; Godden et al., 2008; Husfeldt et al., 2012). Thus, different results reported in Bradley et al. (2018) and the current study results regarding BC in bulk milk of farms using RMS bedding could also be related to sampling methodology used (volume v. weight).

Dairy cows lie down longer in wider stalls compared to narrower stalls and, not surprisingly, wider stalls are also more likely to get dirty (Tucker et al., 2004). Longer stalls (as determined by neck rail position relative to the rear curb) also result in increased time spent standing with all 4 ft in the stall, as well as higher faecal contamination compared to a restrictive neck rail position (Bernardi et al., 2009). Therefore, we hypothesized stall width and length to be associated with BMSCC and BMBC, based on bacteria exposure in the lying stall. Contrary to this hypothesis, wider stalls were associated with lower BMSCC. Further, BMBC was lower on those farms with both wider stalls and longer stall space (i.e. greater distance from neck rail to the back of the stall). It is possible that farms with larger (wider and longer) stalls were more diligent in bedding management, as a weak positive correlation was reported for stall width and how often stalls were scraped. However, no correlation was found between stall length and how often stalls were scraped.

The myriad of differences in both facility design and management practices between tie-stall barns and free-stall barns likely contributed to the differences in percentage point of cows with dirty upper legs and flanks between these two types of systems (Cook, 2002). However, system comparisons of this type are challenging and must be viewed with caution. Additionally, it is important to acknowledge that while these results provide insight in the scope discussed, interpretation must be viewed with caution due to a small sample size of farms, which was further limited once the analysis was completed by housing type. Results included yielded by these analyses do not necessarily include all factors influencing these outcome variables, thus relationships could vary given a larger sample size. Due to the relatively small sample size, there is potential for poor sensitivity, which may have hindered the detection of true associations, including curvilinear effects and interactions. The sample size required to test equivalence and non-inferiority would have been considerably large and not feasible for this particular research. Further, the potential for variation between herds in influencing the analysed outcomes and interactions should not be ignored.

For free-stall barns, the use of an AMS, as compared to a milking parlour, was associated with a higher proportion cows with dirty lower legs. DeVries et al. (2012) reported that nearly 100% of cows in an AMS farm had poor hygiene scores (n = 69 cows). To our knowledge, our findings are the first empirical evidence comparing hygiene in cows milked AMS v. milking parlour. Further research is needed to investigate if management factors (e.g. cow cleaning associated with milking in a parlour) and differences between these milking systems could account for the poorer hygiene reported for AMS farms. A higher proportion of cows with dirty udders and upper legs and flanks were also present in free-stall barns with less frequent alleyways cleaning. These results are in agreement with DeVries et al. (2012),
who reported an association between less frequent alleyways cleaning and proportion of cows with poorer hygiene. A higher proportion of cows with dirty udders and upper legs and flanks were observed in farms with higher DIM. Watters et al. (2013) reported that as DIM increases, cow standing time decreases; thus, stall cleanliness can plausibly affect the cows hygiene. However, stall cleanliness was not assessed in the current study, although it is plausible this relationship could not be confirmed in our study.

For tie-stall barns, a higher proportion of cows with dirty lower legs was observed on farms with access to pasture. Contrary to our results, Popescu et al. (2013) reported no differences in proportion of cows with dirty lower legs, udder, upper legs and flanks between farms where cows had access to pasture, compared to those farms without pasture access. In a study aimed to identify risk factors associated with poor hind leg hygiene in Danish loose housed farms \(n = 42\), a higher proportion of cows with dirty hind legs for farms without access to pasture was reported when compared to those that had access to pasture (Nielsen et al., 2011). However, the researchers in that study noted that the effect of pasture access on cow hind legs cleanliness could be a seasonal effect. It is possible for cows to get dirty while walking on pasture, especially if pasture conditions and the path to the pasture are not ideal (Ellis et al., 2007). Further, it is possible that there is a seasonal impact (i.e. early v. end of grazing season due to weather patterns and path integrity, and potential impact on cow hygiene) as cows have been reported to become dirtier when transitioning from summer grazing to winter housing (Ellis et al., 2007). However, in the present study, no associations were reported between hygiene of lower legs and average temperature nor hygiene of lower legs and relative humidity of the environment. It cannot be discounted that stalls from cows having access to pasture could have been dirtier if producers potentially relied on cows access to pasture for a clean lying area, thus stall cleaning could have been less intense. However, stall cleaning frequency was not retained in the final multivariable of factors associated with lower legs hygiene. Tie-stall farms that utilized lime in their bedding tended to have a higher percentage of cows with dirty lower legs. Causality regarding the link between poor hygiene and lime could not be addressed in this study. Wolfe et al. (2018) reported no treatment effects in hygiene scores for bedding with or without lime added. A qualitative, participatory, approach to assess producers’ motivations for certain bedding management practices, such as adding lime, could bring insight regarding the findings in this study.

It is noteworthy, across all statistical models constructed in this study, that the covariance parameter estimates were, in general, quite high for the herd component and very low for the visit component. High estimates for the herd component would reflect a high amount of herd-to-herd variation in the measured outcomes, as would be expected given the range of characteristics of the farms utilized with this study. The low estimates for the visit component of the models are somewhat surprising, as high consistency between visits would not necessarily be expected for all outcomes measured. This may be related to the fact that the few number of visits did occur close in time (three visits over a 15-day period). These low estimates may also reflect poor model validity, however, based on residual plots model fit was adequate across analyses.

Conclusions

In this study, elevated BC was found in samples of used bedding across bedding types including straw, which has not been compared to other commonly used bedding types in any recent study. Results of this study provide evidence that bedding management can have a profound impact on cow milk quality, bacterial concentrations in the bedding substrates and cow hygiene. High % DM in used bedding plays an important role in reducing BC Streptococcus spp. in used bedding, while low % DM in used bedding is associated with lower milk quality in bulk tank milk. Further, management practices such as frequent addition of bedding have associated benefits for lower BCs in bedding and cow hygiene. Additionally, factors of housing, milking systems and stall design are associated with cow hygiene.

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Declaration of interest

The authors declare no conflict of interest.

Ethics statement

Animal use and study design were approved by the University of Guelph’s Animal Care Committee (AUP#3140) and Research Ethics Board (REB#14JN019), respectively, and animal use complied with the guidelines of the Canadian Council on Animal Care (2009).

Software and data repository resources

None of the data were deposited in an official repository.
**Supplementary material**

To view supplementary material for this article, please visit [this link](http://www.10.1017/S1751731119002787).

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Bacterial count in different bedding types


