Methods of processing recycled manure solids bedding on Midwest dairy farms II: Relationships between bedding characteristics and bedding bacterial count

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Abstract

The first objective of this study was to identify bedding characteristics associated with bedding bacterial count (BBC) in ready-to-use (RTU) recycled manure solids (RMS). The second objective was to explore the association between processing method and bedding characteristics in RTU solids. A third objective was to investigate factors associated with BBC in used solids (after placed in stalls). This observational study recruited 29 premises in Minnesota and Wisconsin. Farms were visited twice, once in August of 2019 and again in January of 2020. At each visit, the herd manager/owner completed a questionnaire describing facilities, bedding and manure management, and mastitis control practices. Additionally, pre-processed RTU and used bedding samples were collected for determination of BBC (coliforms, *Klebsiella* spp., *Streptococcus* or *Streptococcus*-like organisms and *Staphylococcus* spp.) and bedding characteristics (dry matter [DM]), organic matter [OM], water soluble carbohydrates, total nitrogen, soluble nitrogen and pH). Mixed linear regression showed that increased DM, decreased OM and RMS processing method were all associated with reduced BBC in RTU RMS samples. Processing method for RMS was associated with DM, total nitrogen and soluble nitrogen, but not other characteristics in RTU RMS samples. Low BBC in RTU solids, decreased OM in RTU solids, and use of deep bedding systems were associated with lower BBC in used RMS. Implications for these findings are that producers should strive to put clean and high DM fresh (RTU) bedding into stalls. Monitoring BBC and DM in RTU solids may be of value for producers using RMS bedding.

Key words: recycled manure solids bedding, bedding characteristics, bedding bacterial count

Introduction

The popularity of recycled manure solids (RMS) bedding has increased on dairy farms in recent years due to perceived advantages such as reduced costs and increased availability compared to other commonly used bedding materials. Previous studies revealed that, on average, herds using RMS bedding often observed increased bedding bacterial count (BBC) and impaired udder health as compared to herds using other organic or inorganic bedding materials.[1-3] However, variation exists as some herds using RMS are able to achieve low BBC and good udder health.[3] One possible explanation for this apparent success in some RMS herds may be attributed to methods of RMS processing. While many Midwest dairy herds use green (or raw) solids (GRN), other herds employ secondary processing methods, alone or in combination, in an effort to reduce BBC in ready-to-use (RTU) solids. These include, but are not limited to, anaerobic digesters (DIG), mechanical hot air dryers (DRY), and drum composters (COM). In a companion manuscript, we identified that farms using COM or DRY solids bedding had a lower BBC in RTU solids, improved udder health and, for DRY, improved milk production, as compared to herds using either DIG or GRN solids bedding.[4] However, the biological explanation for why some RMS processing systems seem to be more effective than others in reducing BBC requires further investigation.

One obvious mechanism for how secondary processing techniques could reduce BBC could be the direct effect of heating to reduce bacterial viability.[5] However, it is also possible that additional indirect mechanisms are involved in reducing BBC (in either unused or used bedding), with possibilities including increased DM or alteration of other bedding characteristics such as pH or availability of nutrients/substrates to support bacterial growth.[3,6,7] As such, it is important to understand which bedding characteristics are important factors associated with BBC in either unused or used bedding and, of those, which are altered by the processing systems being studied.

Additionally, we need a better understanding of whether (and how) bedding characteristics of RTU bedding, along with bedding management after placing fresh solids into stalls, impact BBC in used bedding from stalls.[3,8–10] The first objective of this study was to investigate the relationship between various bedding characteristics and BBC in RTU RMS. We hypothesized that reduced DM, reduced pH, or increased abundance of certain nutrients that may support bacterial growth (e.g., organic matter [OM], total nitrogen, soluble nitrogen and water-soluble carbohydrates) would be associated with an increased BBC in RTU solids. Our second objective was to examine the association between processing method and bedding characteristics and, for farms using secondary processing systems (COM and DRY), explore whether these bedding characteristics changed after the final processing system (e.g., pre- vs. post-COM or pre- vs. post-DRY). We hypothesized that DRY and COM systems would produce changes in those bedding characteristics that were identified in Objective 1 as being associated with lower BBC in RTU solids. Our third objective was to investigate whether bedding characteristics of RTU solids bedding or manure management practices in the free-stall barn were associated with BBC in used solids (after placed in stalls). We hypothesized that bedding characteristics of RTU solids (e.g., increased DM) as well as management practices (e.g., management of bedding in stalls, manure management, barn characteristics) would be associated with BBC in used solids collected from stalls.

Materials and methods

Herd inclusion criteria

This study enrolled moderate-to-large (> 400 milking cows) free-stall dairy herds in the upper Midwest (Minnesota, Wisconsin) that used RMS bedding. Herds were selected by using convenience sampling to represent four RMS processing systems (GRN, COM, DIG and DRY).

Sample and data collection

Enrolled premises were visited twice, once in August-September of 2019 and again in January 2020. At each visit, in addition to collecting bedding samples, the owner or herd manager completed a questionnaire describing the housing system, bedding and manure management, milking procedures, and routine mastitis control practices (Table 1).

Bedding material sampling

New (unused) samples

Bedding samples collected in herds with GRN or DIG RMS included 1 sample after exiting screw press (RTU solids). For farms using secondary processing systems (COM and DIG), 2 samples were collected; first after exiting the screw press (pre-processed solids) and a second post-pressed sample after exiting the drum composter or drier (RTU solids). Sampling was performed by the study technician wearing clean disposable gloves. In the case of pre-processed solids, 15 samples of pressed RMS were randomly grabbed from the conveyor

Table 1: Herd management information characteristics collected in the questionnaire relevant to the present manuscript.

Management category	Factors described
Herd size	Number of milking cows
Facilities characteristics	Total number of pens Rows per pen Stall surface (deep-bedding/shallow-bedding [concrete, mattress, mixed]) Ventilation type (natural/cross-ventilated/tunnel-ventilated) Ventilation quality (good/poor or fair)
Recycled manure solids management	Type of processing system If digester is used: Temperature (°F) Flow rate (days) If composter is used: Temperature (°F) Flow rate (hours) If dryer is used: Temperature (°F) Flow rate (minutes) Time using current system (years) Storage duration (days)
Bedding management	Bedding depth (inches) Frequency of scraping manure from stall surface Frequency adding new bedding material Frequency scraping alleyways If deep bedding: Frequency removing bedding (regularly/infrequently/never) Use of bedding conditioner (yes/no) If bedding conditioner is used: Type of bedding conditioner Frequency of applying bedding conditioner Site where bedding conditioner is applied

system transporting solids to the dryer or composter. Ready-to-use samples were randomly grabbed from the top 5 cm of bedding from 15 random locations in the pile.

Used samples from stalls

Used bedding samples from all processing systems were collected once during winter 2020. Used bedding samples were collected from the top 5 cm of bedding in the back one-third of 15 randomly selected stalls, representing up to 5 lactating pens.

Sample handling

For each type of sample collected (i.e. pre-processed solids; RTU solids; used solids from stalls), the samples were placed into separate clean dry buckets and mixed thoroughly by hand. The mixed bedding was transferred into a clean one-quart Ziploc® bag. When the bag was at least 3/4 full, air was squeezed out and the bag sealed. Each sample was labeled with farm ID, date, processing type and sample type (pre-processed/RTU/used). For RTU samples, the storage duration of the bedding samples (interval between final processing and sample collection [days]) was recorded. Bedding sample bags were immediately placed on ice after collection and frozen at -20 °C within 8 hr of collection. Samples were later shipped, on ice, to the College of Veterinary Medicine, University of Minnesota, for analysis.

Laboratory characterization of bedding samples

Bedding culture

At the Laboratory for Udder Health (University of Minnesota Veterinary Diagnostic Laboratory, Saint Paul, MN), frozen bedding samples were thawed to room temperature and a subsample subjected to routine culture procedures as previously described.[3] Briefly, 50 cm^3 of packed bedding material was transferred into a new Whirl-Pak bag, mixed with 250 mL of sterile water (Becton Dickinson and Company, Franklin Lakes, NJ), and left to stand for 10 min. Serial 10-fold dilutions of the samples were made using sterile water. Sample dilutions were plated onto MacConkey agar (Gram-negative bacteria selection) and colistin naladixic acid agar (Gram-positive bacteria selection, Becton Dickinson and Company) plates and incubated overnight at 37 °C. For the MacConkey plates, lactose fermenting (pink) colonies were counted as coliform bacteria and all other colonies were counted as non-coliform Gram-negative bacteria. Colonies with a confluent appearance on MacConkey agar were identified to the genus level using a MALDI Biotyper (Bruker Daltonics, Billerica, MA) and colonies identified as *Klebsiella* spp. were counted and reported as a percentage of total coliform count. For colistin naladixic acid plates, colony morphology in conjunction with catalase reaction and Gram stain were used to differentiate colonies of *Staphylococcus* spp., *Streptococcus* spp. and *Streptococcus*-like organisms (SSLO), and *Bacillus* spp. Total bacteria count (TBC) and counts of *Bacillus* spp., coliforms, *Klebsiella* spp., non-coliform Gram-negatives, *Staphylococcus* spp., and SSLO were recorded as colony-forming units per cubic centimeter of wet bedding. The minimum limit of detection was 25 colony-forming units (CFU)/cm^3.

Bedding characteristics

After subsampling for culture, bedding samples were split into two aliquots and immediately refrozen (–20 °C). One aliquot was submitted on ice to the Dairy One Forage Laboratory (Ithaca, NY) for determination of bedding characteristics (dry matter [DM, %], ash [%], total nitrogen [%], soluble nitrogen [%], and water-soluble carbohydrates [%]) using wet chemistry analysis. Organic matter (OM, %) was calculated as 1 - % ash. The second aliquot was analyzed for pH.[3]

Statistical analysis

Data were entered into an Excel file (Microsoft Corp., Redmond, WA) and statistical analyses completed using R (**https://www.r-project.org/**; version 4.3.2). For the analysis, each farm served as the unit of observation, with 2 observations (farm visits) available, except for 1 farm and the used RMS samples, which were collected only once. Summary statistics included mean and standard deviation for numerical outcomes and proportions for categorical outcomes and were generated using Table 1 package in R.[11] Before modeling, data was explored to examine distribution and accuracy. Outliers were defined as observations that lied 3 or more standard deviations away from the mean. If outliers were discovered, paper records were utilized to correct any discrepancies or data-entry errors. No outliers were dropped from the analysis. For exploratory analysis, univariable analyses assessing the association between processing system (explanatory variable) and herd characteristics (dependent variables) were carried out using linear regression for numerical outcomes and Fisher's exact test for categorical dependent variables. Unconditional associations between the type of final processing system, bedding characteristics and BBC in pre-processed RTU and used solids were explored using mixed linear regression as implemented in the "lme4" package.[12] The "emmeans" package in R was used to compute the estimated marginal means.[13] Multiple comparisons were accounted for when necessary using the Tukey method in "emmeans" package.[13] Farm-ID was included as a random intercept when appropriate to account for repeated sampling in each farm.

Objective 1. Relationship between bedding characteristics and BBC in RTU RMS bedding

The BBC were log (base 10) transformed before data analysis to normalize the data distribution and reduce skewness. Mixed linear regression was used to investigate the association between bedding characteristics (DM %, OM %, total nitrogen %, soluble nitrogen %, water soluble carbohydrates %, pH; explanatory variables) and BBC in RTU solids (coliform, SSLO, *Staphylococcus* spp. and *Klebsiella* spp. counts; dependent variables). Season, storage duration (days) prior to sample collection, herd size and pre-processing system were offered as potential confounders into the models. Final processing system was forced into all the models.

Objective 2. Relationship between processing system and bedding characteristics in RMS bedding

Associations between the processing system (DRY/COM/DIG/GRN; explanatory variable) and each bedding characteristics in RTU samples (DM, OM, pH, total nitrogen, soluble nitrogen, water soluble carbohydrates; dependent variables) were investigated using mixed linear regression. For farms using secondary processing systems (DRY or COM), a secondary analysis including only the DRY and COM samples was completed to explore if changes in bedding characteristics occurred when comparing pre- vs post-COM or pre- vs post-DRY samples. The latter models offered the sample type (pre or post processing),

processing system (DRY/COM) and an interaction term. Season, storage duration (days) prior to sample collection, herd size and pre-processing system (Objective 2 only) were offered as confounders into the models.

Objective 3. Investigation of factors associated with BBC in used RTU collected from stalls

The investigation of factors associated with BBC in used solids collected from stalls (dependent variable) was assessed using mixed linear regression. Variables offered to the models (potential explanatory variables) included BBC in RTU solids (forced), bedding characteristics in RTU solids (DM, OM, pH, total nitrogen, soluble nitrogen, water soluble carbohydrates), herd size, season, housing system and bedding management.

Model building process (Objectives 1, 2 and 3)

Figure 1 shows a directed acyclic graph with the hypothesized causal pathway and potential confounders. To build multivariable models, variables were offered to models as potential predictors or potential confounders if an initial univariable model showed *P*-value ≤ 0.20. A backward stepwise process was used to remove covariates with high *P*-values until they reached a *P*-value ≤ 0.10. The normality assumption was tested by examining the distribution of the model's residuals and using quantile-quantile plots. The linearity assumption was assessed by the visualization in a scatterplot of the association between the explanatory and dependent variables. Additionally, linearity was further tested by adding a quadratic term which was maintained in the model when *P* ≤ 0.05. Homoscedasticity was evaluated by plotting the observed residuals vs. the predicted values. Effect modification on the additive scale was tested when it was biologically plausible and interaction terms were kept in the models if *P* ≤ 0.05. Statistical significance was set at *P* ≤ 0.05.

Results

Herd characteristics

This observational study enrolled 29 free-stall premises from Minnesota (n = 8) and Wisconsin (n = 21). Among the enrolled premises, 7 used GRN, 4 used COM, 6 used DIG solids and 12 used DRY solids. One of the GRN premises was visited only once (summer) while all other premises were visited twice (summer/winter). Because 1 of the DRY premises transported RTU bedding to a second premise within the same farm system, only 1 (not 2) bedding sample observations per season were included in the analysis for this farm system. Of the 12 premises using DRY solids, 4 used a dryer as the sole processing step, while 8 used some combination of 2 processing steps: 7 processed slurry through an anaerobic digester prior to drying the pressed solids, and 1 pressed solids through a mechanical composter prior to drying. However, because preliminary analysis showed no important difference in outcomes when comparing single-step vs. 2-step systems (results not reported), the observations from all 12 premises were combined into the DRY category for the final analysis.

Descriptive characteristics for enrolled herds are shown in Table 2. The mean ± SD number of milking cows was lower in GRN (886 ± 430) or COM (633 ± 318) compared to DIG (2,930 ± 1,300) or DRY premises (3,490 ± 1,740). Storage duration (days) of RTU solids was 2.7 ± 5.2 days, ranging between 0 and 21 days, with no differences across processing systems (*P* = 0.53). Overall, farms added new material to stalls an average of 3.7 ± 2.1 times per week, though farms using GRN (5.5 ± 0.7) added new bedding to stalls more frequently than other processing systems (COM: 3.7 ± 1.1, DIG: 3.0 ± 0.7, DRY: 2.9 ± 0.6; *P* = 0.04). The overall (mean ± SD) frequency of cleaning contaminated bedding from the backs of stalls was 2.7 ± 0.7 times per day, and occurred more frequently in DRY (2.9 ± 0.3) than COM (1.7 ± 1.5) and DRY (2.9 ± 0.3) (*P* = 0.05). Farms reported a predominance of natural (vs. mechanical) ventilation systems (66.7 %) (*P* = 0.87) and use of deep bedded (vs. shallow) stalls (70.4 %) (*P* = 0.87). The average bedding depth in stalls was 8.6 (4.4) inches and did not differ across the 4 processing system types (*P* = 0.61).

Figure 1: Hypothesized directed acyclic graph of the causal pathway between recycled manure solids processing system, bedding characteristics and bedding bacterial count.[a] Potential predictors categories included: Bedding characteristics in RTU solids (dry matter, organic matter, total nitrogen, soluble nitrogen, water soluble carbohydrates, pH), Season (summer, winter), Herd size (number of milking cows), Facilities characteristics (rows per pen [2 vs. 3 rows]), Stall surface (deep bedding, shallow bedding), Ventilation type (natural, other), Ventilation quality (fair, good), Bedding management (Bedding depth [cm], frequency scraping manure back of stall surface [times per day], frequency of adding new bedding material [times per week], frequency of scraping alleyways [times per day], bedding conditioner [Y/N]).

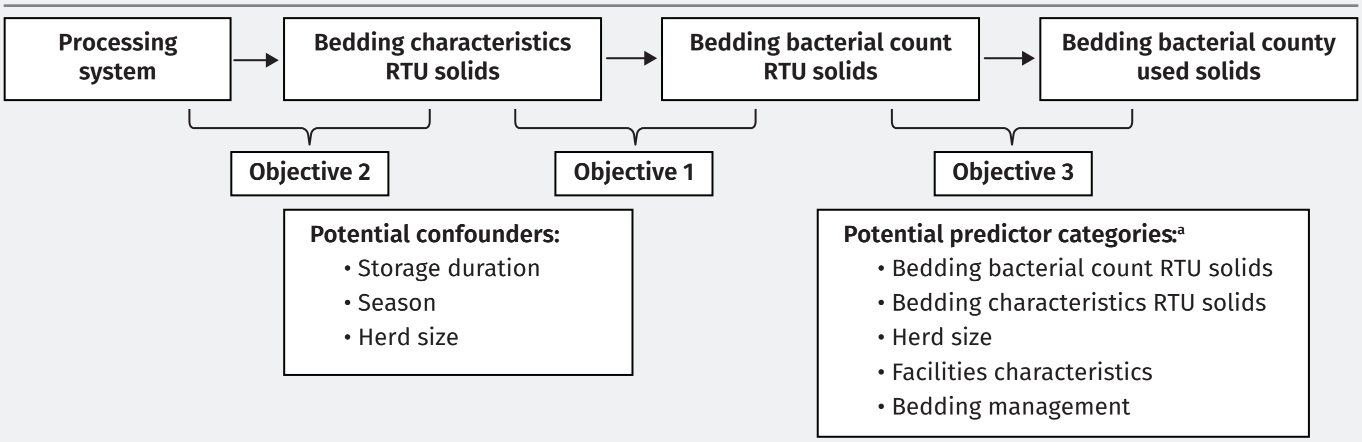

Table 2: Descriptive statistics of enrolled herds by final processing system (n = 29). Numerical variables are represented by mean ± SD and categorical variables by n (%).

Item	Green (n = 7)	Composted (n = 4)	Digested (n = 6)	Dried (n = 12)	Overall (n = 29)	*P*-value
Herd size (n milking cows)	886 ± 430[a]	633 ± 318[ai]	2930 ± 1300[bii]	3490 ± 1740[b]	2370 ± 1750	< 0.001
Days storage (days)	0.9 ± 0.4	1.3 ± 0.5	4.8 ± 8.0	3.3 ± 5.8	2.7 ± 5.2	0.53
Freq. add bedding stalls (times / week)	5.5 ± 0.7[ai]	3.7 ± 1.1[aii]	3.0 ± 0.7[ab]	2.9 ± 0.6[b]	3.7 ± 2.0	0.04
Freq. cleaning stalls (times / day)	2.6 ± 0.8[ab]	1.7 ± 1.53[a]	2.8 ± 0.41[ab]	2.9 ± 0.3[b]	2.7 ± 0.7	0.05
Freq. cleaning alleyways (times / day)	5.5 ± 2.3	3.7 ± 3.1	3.0 ± 1.4	2.9 ± 1.3	4.8 ± 4.4	0.60
Bed depth (inches)	7.4 ± 3.5	6.3 ± 3.1	9.7 ± 3.3	9.3 ± 5.6	8.6 ± 4.4	0.61
Free stall surface						0.87
Deep bedding	4 (57.1%)	2 (75.0%)	5 (83.3%)	8 (72.7%)	19 (70.4%)	
Shallow bedding *	3 (32.9%)	1 (25.0%)	1 (16.7%)	3 (27.3%)	8 (29.6%)	
Use of bedding conditioner						0.45
No	6 (85.7%)	2 (50.0%)	3 (50.0%)	6 (50.0%)	17 (58.6%)	
Yes	1 (14.3%)	2 (50.0%)	3 (50.0%)	6 (50.0%)	12 (41.4%)	
Ventilation type						0.60
Natural	6 (85.7%)	3 (100.0%)	3 (50.0%)	6 (60.0%)	18 (66.7%)	
Other †	1 (14.3%)	0 (0%)	3 (50.0%)	4 (40.0%)	9 (33.3%)	
Ventilation quality						0.71
Fair or poor	2 (14.3%)	0 (0%)	0 (0%)	2 (18.2%)	4 (14.8%)	
Good	5 (85.7%)	3 (100%)	6 (100%)	9 (81.8%)	23 (85.2%)	

[a,b] Means within a row with an uncommon superscript differ $P \leq 0.05$.

[i,ii] Means within a row with an uncommon superscript indicate $0.05 < P \leq 0.10$.

* Shallow bedding: Concrete, mattress or mixed systems.

† Ventilation type: Other: Cross-ventilated, tunnel-ventilated or mixed ventilation systems.

Objective 1. Associations between bedding characteristics and bedding bacterial count in RTU solids

Our first objective investigated the association between bedding characteristics (explanatory variable) and BBC (dependent variable) in RTU solids (Table 3). Although BBC was measured in 56 RTU bedding samples (GRN = 13, COM = 8, DIG = 12, DRY = 23), bedding characteristics results were only available for 48 bedding samples (GRN = 13, COM = 6, DIG = 11, DRY = 18). In multivariable regression models, DM % and OM % were the only bedding characteristics associated with BBC. Specifically, increased DM (%) was statistically or numerically associated with reduced counts of coliforms (Estimate ± SE: -0.05 ± 0.03, $P = 0.08$), SSLO (-0.08 ± 0.02, $P = 0.003$) and *Staphylococcus* spp. (-0.06 ± 0.03, $P = 0.08$). Increased organic matter (%) was associated with increased coliform counts (positive nonlinear association) (linear term: 14.95 ± 6.41, $P = 0.02$; quadratic term: -0.08 ± 0.04, $P = 0.03$) and increased counts of *Staphylococcus* spp. (0.31 ± 0.14, $P = 0.03$). Other bedding characteristics (water soluble carbohydrates, total nitrogen, soluble nitrogen and pH) were not associated with BBC in RTU solids. Final processing system, which was forced into the models, was associated with BBC as counts of coliforms and SSLO were reduced in COM samples, and counts of *Klebsiella* spp. were reduced in DRY, COM and DIG RTU samples ($P < 0.05$).

Objective 2. Association between processing system and bedding characteristics in ready-to-use solids

For Objective 2 we explored if RMS final processing systems (explanatory variable) were associated with bedding characteristics (dependent variables) in RTU solids (Table 4). In addition to all RTU samples tested, bedding characteristics were also determined in 20 pre-processed samples (pre-COM = 5, pre-DRY = 15). Results showed that DM (%) (Estimated marginal mean ± SE) was greater in DRY (44.26 ± 2.08) compared to GRN (30.76 ± 2.48), COM (30.27 ± 3.60) or DIG (32.49 ± 2.70) RTU solids ($P < 0.001$). DIG solids had higher total nitrogen (%) (2.41 ± 0.07) as compared to GRN (1.61 ± 0.07), COM (1.77 ± 0.09) or DRY (1.55 ± 0.05) solids ($P = 0.01$). Following a similar pattern to total nitrogen, soluble nitrogen (%) was numerically or statistically higher in DIG (0.49 ± 0.05) compared to GRN (0.21 ± 0.04, $P = 0.002$), COM (0.27 ± 0.07, $P = 0.06$) or DRY solids (0.34 ± 0.04, $P = 0.09$). No evidence of an association was found between processing method and OM (%) ($P = 0.26$), water soluble carbohydrates (%) ($P = 0.20$) or pH ($P = 0.45$).

In a secondary analysis of only the DRY and COM samples, mixed linear regression was used to describe changes in bedding characteristics in pre- vs. post-COM or pre- vs. post-DRY samples (Table 5). Dry matter (%) increased significantly after processing in DRY samples (Estimated marginal means ± SE pre: 29.68 ± 2.28 vs. post: 44.43 ± 2.08, $P < 0.001$), but remained unchanged after processing in COM samples (pre: 31.73 ± 3.99 vs. post: 30.49 ± 3.59, $P = 0.81$). No differences in any other bedding characteristics (OM, pH, total nitrogen, soluble nitrogen and water-soluble carbohydrates) were observed when comparing pre- vs. post-DRY or pre-vs. post-COM samples ($P > 0.05$).

Objective 3. Factors associated with BBC in used RMS bedding collected from stalls

Our third objective was to identify factors associated with BBC in used solids (Table 6). For this investigation, we had 26 sets of samples wherein we knew BBC and bedding characteristics

Table 3: Final multivariable regression models describing the relationship between bedding characteristics and bedding bacterial count in ready-to-use solids (n = 48).

Dependent variables	Explanatory variables*	Estimate ± SE	*P*-value
Model 1. Coliforms ($\log_{10}$ CFU/cm3)	DM	-0.05 ± 0.03	0.08
	OM	14.95 ± 6.41	0.02
	OM 2	-0.08 ± 0.04	0.03
	FPS: Green	Referent[a]	0.03
	FPS: Composted	-2.07 ± 0.73[b]	
	FPS: Digested	-1.27 ± 0.64[ab]	
	FPS: Dried	-1.48 ± 0.66[ab]	
Model 2. SSLO ($\log_{10}$ CFU/cm3)	DM	-0.08 ± 0.02	0.003
	FPS: Green	Referent[a]	< 0.001
	FPS: Composted	-3.52 ± 0.73[b]	
	FPS: Digested	-0.62 ± 0.63[a]	
	FPS: Dried	-1.12 ± 0.63[a]	
Model 3. *Staphylococcus* spp. ($\log_{10}$ CFU/cm3)	DM	-0.06 ± 0.03	0.08
	OM	0.31 ± 0.14	0.03
	FPS: Green	Referent	0.78
	FPS: Composted	-0.61 ± 1.08	
	FPS: Digested	-0.38 ± 0.97	
	FPS: Dried	-0.93 ± 0.91	
Model 4. *Klebsiella* spp. ($\log_{10}$ CFU/cm3)	FPS: Green	Referenta	< 0.001
	FPS: Composted	-1.87 ± 0.36[b]	
	FPS: Digested	-1.76 ± 0.32[b]	
	FPS: Dried	-1.88 ± 0.28[b]	
	Season: Summer	Referent	0.06
	Season: Winter	-0.41 ± 0.21	

[a,b] Estimates within a model with an uncommon superscript differ $P \leq 0.05$.
Multiple comparisons were accounted for when necessary using the Tukey method.
* Final processing system (FPS) was forced into all the models.
CFU: Colony-forming units
DM: Dry Matter (%)
OM: Organic Matter (%)
TN: Total Nitrogen (%)
[2] Quadratic term.

Table 4: Final Mixed multivariable regression models assessing the association between final processing system and bedding characteristics in ready-to-use solids (n = 48).

Dependent variables	Explanatory variables	EMM ± SE	Estimate ± SE	*P*-value
Model 1. DM (%)	FPS: Green	30.76 ± 2.48^{a}	Referent	< 0.001
	FPS: Composted	30.27 ± 3.60^{a}	-0.49 ± 4.34	
	FPS: Digested	32.49 ± 2.70^{a}	1.73 ± 3.65	
	FPS: Dried	44.26 ± 2.08^{b}	13.50 ± 3.22	
Model 2. OM (%)	FPS: Green	91.03 ± 0.77^{a}	Referent	0.26
	FPS: Composted	91.38 ± 0.99^{a}	0.34 ± 1.24	
	FPS: Digested	89.72 ± 0.80^{a}	-1.31 ± 1.12	
	FPS: Dried	91.72 ± 0.58^{a}	0.70 ± 0.99	
	Storage duration (days)	–	-1.18 ± 0.48	0.02
	Storage duration (days) 2	–	0.05 ± 0.02	0.02
Model 3. WSC (%)	FPS: Green	1.00 ± 0.09^{a}	Referent	0.20
	FPS: Composted	1.23 ± 0.14^{a}	0.23 ± 0.15	
	FPS: Digested	0.85 ± 0.09^{a}	-0.15 ± 0.13	
	FPS: Dried	1.00 ± 0.08^{a}	0.001 ± 0.14	
	Herd size (per 100 cows)	–	-0.01 ± 0.004	0.02
Model 4. TN (%)	FPS: Green	1.61 ± 0.07^{a}	Referent	< 0.001
	FPS: Composted	1.77 ± 0.09^{a}	0.16 ± 0.09	
	FPS: Digested	2.41 ± 0.07^{b}	0.80 ± 0.07	
	FPS: Dried	1.54 ± 0.05^{a}	-0.07 ± 0.09	
	PPS: No	1.52 ± 0.03	Referent	< 0.001
	PPS: Yes	2.14 ± 0.08	0.62 ± 0.09	
Model 5. SN (%)	FPS: Green	0.21 ± 0.04^{a}	Referent	0.001
	FPS: Composted	0.27 ± 0.07^{ab,i}	0.06 ± 0.08	
	FPS: Digested	0.49 ± 0.05^{b,ii}	0.28 ± 0.06	
	FPS: Dried	0.34 ± 0.04^{ab}	0.13 ± 0.06	
Model 6. pH	FPS: Green	8.89 ± 0.08^{a}	Referent	0.45
	FPS: Composted	8.93 ± 0.10^{a}	0.04 ± 0.12	
	FPS: Digested	8.87 ± 0.08^{a}	-0.02 ± 0.11	
	FPS: Dried	8.77 ± 0.06^{a}	-0.12 ± 0.10	
	Season: Summer	9.02 ± 0.04^{a}	Referent	< 0.001
	Season: Winter	8.72 ± 0.04^{a}	-0.30 ± 0.03	

[a,b] Means within a column with an uncommon superscript differ $P \leq 0.05$.
[i,ii] Means within a row with an uncommon superscript indicate $0.05 < P \leq 0.1$.
Multiple comparisons were accounted for when necessary using the Tukey method.
EMM: Estimated marginal means
FPS: Final processing system
PPS: Pre-processing system
DM: Dry Matter
OM: Organic Matter
WSC: Water-soluble carbohydrates
TN: Total Nitrogen
SN: Soluble Nitrogen
Different letters indicate significant differences between processing systems.
[2] Quadratic term.

Table 5: Results from a mixed linear regression model assessing the association between processing system and change in bedding characteristics between pre-processed and RTU samples (n = 44).

Dependent variable	FPS	Pre (EMM ± SE)	Post (EMM ± SE)	*P*-value	Across systems	
					Estimate ± SE	*P*-value
Model 1. DM (%)	Dried	29.68 ± 2.28	44.43 ± 2.08	< 0.001	Referent	0.01
	Composted	31.73 ± 3.99	30.49 ± 3.59	0.81	-15.99 ± 5.99	
Model 2. OM (%)*	Dried	91.48 ± 0.62	91.56 ± 0.62	0.71	Referent	0.23
	Composted	91.61 ± 1.08	91.13 ± 1.07	0.24	-0.56 ± 0.45	
Model 3. WSC (%)†	Dried	0.90 ± 0.08	0.96 ± 0.07	0.55	Referent	0.32
	Composted	1.24 ± 0.14	1.10 ± 0.14	0.43	-0.20 ± 0.20	
Model 4. TN (%)	Dried	1.67 ± 0.10	1.62 ± 0.10	0.51	Referent	0.92
	Composted	1.47 ± 0.18	1.44 ± 0.17	0.79	0.01 ± 0.14	
Model 5. SN (%)	Dried	0.42 ± 0.04	0.33 ± 0.04	0.13	Referent	0.99
	Composted	0.35 ± 0.07	0.27 ± 0.06	0.38	0.002 ± 0.11	
Model 6. pH‡	Dried	8.83 ± 0.07	8.78 ± 0.06	0.44	Referent	0.55
	Composted	8.92± 0.12	8.95 ± 0.11	0.80	± 0.13	

* Controlled for storage duration.
† Controlled for herd size and season.
‡ Controlled for season.
EMM: Estimated marginal means
FPS: Final processing system
DM: Dry Matter
OM: Organic Matter
WSC: Water-soluble carbohydrates
TN: Total Nitrogen
SN: Soluble Nitrogen

Table 6: Results from regression models with the objective of identifying factors associated with bedding bacterial count in used solids (n = 26).

Dependent variable	Explanatory variables*	Estimate ± SE	*P*-value
Model 1. Coliform (log_{10} CFU/cc)	BBC RTU	-0.17 ± 0.14	0.22
Model 2. SSLO (log_{10} CFU/cc)	OM RTU	0.13 ± 0.05	0.03
	FS Surface: Deep	Referent	0.04
	FS Surface: Shallow	0.67 ± 0.31	
	BBC RTU	-0.07 ± 0.09	0.49
Model 3. *Staphylococcus spp.* (log_{10} CFU/cc)	OM RTU	0.32 ± 0.14	0.04
	BBC RTU	0.37 ± 0.17	0.04
Model 4. *Klebsiella* spp. (log_{10} CFU/cc)	BBC RTU	1.41 ± 0.45	0.005

* Bedding bacterial count (BBC) in RTU solids for the corresponding microorganisms was forced in all models.
CFU: Colony forming units
BBC: Bedding bacterial count
RTU: Ready-to-use
OM: Organic matter
FS surface: Free-stall surface

in RTU samples as well as BBC in used samples collected from the same farm on the same day (GRN = 6, COM = 3, DIG = 6, DRY = 11). In particular, we were interested in whether BBC or bedding characteristics of the RTU solids (before placing in stalls) or herd management practices (e.g. facility design, bedding or manure management) were associated with BBC in used bedding from stalls. In multivariable models, OM (%) in RTU solids was positively associated with SSLO (CFU/cm^3) (Estimate (SE): 0.13 (0.05), $P = 0.03$) and *Staphylococcus* spp. counts (0.32 ± 0.14, $P = 0.04$) in used samples. Bedding BC in RTU solids had a positive association with counts of *Staphylococcus* spp. (Estimate ± SE: 0.36 ± 0.17, $P = 0.04$) and *Klebsiella* spp. (1.32 ± 0.43, $P = 0.006$), but not coliforms (-0.17 ± 0.14, $P = 0.22$) or SSLO (-0.07 ± 0.09, $P = 0.49$) in used solids. Farms with shallow bedding systems had higher SSLO counts in used RMS samples compared to those with deep bedding systems (0.67 ± 0.31, $P = 0.04$).

Discussion

Recycled manure solids constitute an appealing and sustainable bedding material for many dairy farmers. However, raw/fresh RMS represents a potential threat for animal health given the intrinsic contamination of manure with different types of potential pathogens,[14] including those that can lead to mastitis.[3] A previous companion article described that certain secondary processing methods, especially COM or DRY, are associated with reduced BBC and enhanced udder health as compared to GRN solids.[4] However, we did not previously evaluate how this effect was achieved. Was it simply a direct negative effect of heat treatment on bacterium viability, or do these secondary processing methods modify other bedding characteristics which could independently impact BBC in either unused or used samples RMS?

The analysis described in this article improves our comprehension of the different mechanisms that allow different processing systems to reduce BBC, and thus the risk of mastitis. To our knowledge, this is the first epidemiological study that investigated the association between 4 different RMS processing systems, bedding characteristics, and BBC in both RTU and used RMS bedding samples. Study strengths include the enrollment of farms from both Minnesota and Wisconsin that used 4 different processing systems and sampling during summer and winter seasons. Nonetheless, results from this study should be interpreted cautiously as our results should be confirmed with a larger number of farms as well as different geographical regions. An additional limitation is that used bedding samples were only collected in 1 season, thereby limiting our sample size for the third objective. While results may be generalized to other similar climates (e.g., Northeast U.S. or Canada), generalizing our results to hotter or more arid regions of the U.S. (e.g., South, Southwest or West) should be done with caution. The cross-sectional nature of this study means that readers should be cautious in drawing conclusions about causality where associations are detected. Despite our best efforts to account for confounding, it is still possible that other unmeasured factors could affect the findings of this observational study.

Objective 1: Association between bedding characteristics and bedding bacterial count in ready-to-use solids

The finding that coliforms, SSLO and *Staphylococcus* spp. counts in RTU samples were all negatively associated with DM in RTU samples agrees with our previous findings.[7] It has been suggested that the negative association between DM and BBC in RTU solids is totally confounded by the direct effect of heating during secondary processing (i.e., heat treatment could both kill bacteria as well as increase DM %). However, in our analysis, the negative association between DM and BBC remained significant even after controlling for the final processing system. This suggests that DM may independently explain at least some of the differences in BBC. In other words, achieving increased DM is still a goal for RMS, regardless of the type of processing method (GRN, DIG, COM or DRY) used on the farm. On Midwest dairies it has anecdotally been suggested that herds should strive to achieve DM between 35-45% due to concerns that DM exceeding 45-50% may result in excessive dust in barns.

Organic matter in RTU solids was also associated with BBC in RTU solids. Since it is known that OM supports bacterial growth, it is plausible that this is connected to the creation of conditions that favor bacterial growth through changes in OM that could occur with certain types of processing.[6] In fact, 2 of the investigated processing systems (COM and DIG) involve the breakdown of OM by microorganisms to treat RMS, which could theoretically reduce BBC in RTU or used solids.[15-17] Other bedding characteristics (water soluble carbohydrates, total nitrogen, soluble nitrogen and pH) were not associated with BBC in RTU solids but could still conceivably be associated with bacterial proliferation and BBC in used solids (see Objective 3).

After controlling for important bedding characteristics (DM and OM), the final processing system was still associated with counts of coliforms, SSLO and *Klebsiella* spp. in RTU solids. The causal path explaining this effect could theoretically be attributed to the direct effect of heat on microbe viability,[5] though it is always possible that other unmeasured factors are involved.

Objective 2: Association between processing system and bedding characteristics

Earlier analysis in a companion paper[4] showed that processing method was associated with BBC in RTU solids. We wanted to know how this apparent effect might be mediated. An obvious hypothesis is that exposure of solids to high temperatures could compromise the survival of pathogens (similar to heat-treating colostrum or pasteurizing milk).[5,18] However, it is also possible that the processing method could modify certain bedding characteristics which are important to support bacterial growth in either RTU or used solids.

Here and in our previous study,[7] DM was negatively correlated with BBC in RTU solids, as discussed in Objective 1. In addition, DM was higher in DRY solids as compared to other processing systems, as well as in post-DRY compared to pre-DRY bedding samples. These findings could potentially explain why DRY had a lower BBC to GRN and DIG solids.[4] Nonetheless, COM RTU solids had similar BBC to DRY RTU solids, yet showed no evidence of a difference in DM compared to DIG or GRN solids or in response to processing (pre vs. post). This is inconsistent with results from earlier studies in which use of composting has been related to an increased DM.[15,16] These findings suggest that for COM solids, the decrease in BBC of mastitis pathogens in RTU solids may be associated to direct heat exposure rather than a mere increase in DM.

In our study, RTU OM did not vary across processing systems or differ between before and after processing in secondary systems (COM or DRY). This suggests that although OM may have an impact on bacterial growth and BBC, it is unrelated to the type of RMS processing system used and therefore is unlikely to explain why some systems are more effective than others in reducing BBC in RTU RMS. Our observation that total nitrogen and soluble nitrogen were higher in DIG solids compared to other processing systems is in line with previous research.[15] Other factors such as the diet[19]or potential losses of ammonia when samples were dried before determination of nitrogen content,[15] could also have an impact on the observed results. Despite these differences, total nitrogen and soluble nitrogen were not associated with BBC in RTU (Objective 1). Last, final processing system was not associated with water-soluble carbohydrates or pH of RTU solids.

Objective 3: Bedding characteristics and management factors associated with BBC in used solids from stalls

Numerous studies have reported that BBC increase rapidly after fresh bedding is placed into stalls.[6,17,20,21] Some of this effect could be explained by new contamination caused by cows tracking manure and moisture into stalls.[22] Additionally, this rapid increase in BBC could represent proliferation of bacteria that were already present in the fresh bedding and given exposure to suitable conditions (e.g. warmth, moisture, nutrients).[6] In this objective we explored whether BBC in fresh (RTU) bedding, bedding characteristics in RTU bedding (e.g., nutrients or pH), or herd management practices (e.g., ventilation, facility design, bedding management, manure management) were associated with BBC in used RMS samples collected from stalls, with a view to identifying possible mitigation strategies that could help to reduce bacterial proliferation after solids are placed into stalls.

For analysis, BBC in RTU solids were forced into all models since it represents the baseline contamination of RTU by mastitis-causing microorganisms before bedding is placed in the stalls. Bacteria counts in RTU solids were associated with increased counts of *Staphylococcus* spp. and *Klebsiella* spp. counts in used RMS, supporting the notion that we should strive to put clean (low BBC) fresh bedding into stalls.

Organic matter in RTU solids was associated with BBC in used solids in multiple bacterial groups, which could be related to the fact that OM provides nutrient substrates that promote bacterial growth.[6] This has been described in other studies wherein organic bedding materials (e.g. straw, wood shavings, RMS) generally have higher BBC compared to non-organic bedding.[1-3] However, given that OM in RTU solids did not differ among the 4 processing methods evaluated in this study, we may have little ability to modify this through existing processing methods. Future studies should explore if there are other predictors for OM in RTU RMS that could potentially be manipulated by producers to reduce BBC.

Dry matter and other bedding characteristics in RTU solids were not associated with BBC in used solids. However, DM was negatively associated with the BBC in RTU solids, which was subsequently associated with the BBC in used solids. Therefore, our results suggest that to maintain a low BBC in used solids, it is still important for RTU solids to achieve a high DM content. As for other characteristics, total nitrogen, soluble nitrogen, water soluble carbohydrates and pH of RTU solids were not associated with BBC in either RTU or used solids, suggesting that these may not be important for users to monitor or manipulate to control BBC.

As for other facility or herd management practices, the use of a shallow bedding systems was associated with increased BBC in used RMS samples as compared to deep bedding systems. This is consistent with previous reports[15] and could be attributed to the fact that reduced amounts of bedding allow free-stall surfaces to become more quickly contaminated by urine and feces which create beneficial conditions for bacterial growth. It is somewhat surprising that none of the other facility characteristics or bedding or manure management practices investigated in this study were associated with BBC in used RMS bedding. These findings conflict with our earlier observation that good ventilation and increased frequency of scraping contaminated material from the back of stalls was associated with improved udder health outcomes in herds using RMS bedding.[4] This is possibly due to the relatively small number of herds in the current study which limited the precision of the estimates generated. Larger studies are needed for a more thorough evaluation of facility design and management as risk factors for high BBC in used bedding.

Conclusions

This study advances our understanding of the potential mechanisms by which certain RMS processing systems may impact BBC in RTU and used manure solids bedding, and therefore, udder health. Our results suggest that BBC in RTU solids can be reduced by the type of processing system used both directly (e.g., the direct effect of heat in DRY and COM systems may reduce microbe viability), as well as indirectly through increased DM %. Increased DM of RTU bedding should be a goal for producers using RMS bedding, regardless of the processing method used. We also observed that reducing counts of *Klebsiella* spp. and *Staphylococcus* spp. in RTU solids were associated with reduced BBC in used bedding in stalls, supporting the notion that producers should strive to place clean bedding into stalls. Although OM levels were positively associated with BBC in RTU and used RMS, OM levels were not impacted by the RMS processing methods we evaluated, and therefore may not be useful to monitor or attempt to manipulate. Similarly, other bedding characteristics (e.g., various nutrient contents and pH) were not affected by processing method, nor associated with BBC in either RTU or used solids, and as such may not be of value to monitor or attempt to manipulate, at least as relates to attempting to reduce BBC and improving udder health.

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Conflicts of interest

The authors do not have any conflicts of interest to declare.

Author contributions

Dr. Peña-Mosca contributed to acquisition of data, analysis and interpretation, drafting of the manuscript, and approval of the final version to be published. Dr. Godden contributed to conception and design, herd enrollment, drafting of the manuscript, and approval of the final version to be published. Dr. Royster, Dr. Crooker and J. Timmerman contributed to conception and design, acquisition of data, manuscript revision and approval of the final version to be published.

Abbreviations

BBC	Bedding bacteria count
COM	Composted
DIG	Digested
DRY	Dried
DM	Dry matter
GRN	Green
OM	Organic matter
RMS	Recycled manure solids
RTU	Ready-to-use
SSLO	*Streptococcus* spp. and *Streptococcus*-like organisms

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