Comparison of number of *Streptococcus uberis* calculated on a volume or weight basis in sand and sawdust bedding

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Objective—To determine a method for comparing counts of *Streptococcus uberis* in sand and sawdust and account for the influence of weight or volume of the bedding material.

Sample Population—2 sources of kiln-dried sawdust and 2 sources of washed sand.

Procedures—Sterilized bedding material (100 ml) was weighed and uniformly distributed in an aluminum pan. Each sterilized bedding material was inoculated with a mean of 3.6×10^6 (experiment 1) or 2.4×10^7 (experiment 2) colony-forming units (CFU) of *S uberis*/ml of bedding material. Without allowing time for replication of *S uberis*, inoculated bedding materials were washed with sterile saline (0.9% NaCl) solution. A 200-ml aliquot of wash solution was serially diluted up to 2,500 times with additional saline solution and inoculated on plates containing tryptose agar with 5% sheep blood. After incubation for 48 hours, number of CFU of *S uberis* was counted. This procedure was replicated 19 and 16 times for each bedding material in experiments 1 and 2, respectively.

Results—Evaluation of Bonferroni 95% confidence intervals revealed significant differences for counts of *S uberis* calculated on a weight basis between sand and sawdust.

Conclusions and Clinical Relevance—Comparison of counts of *S uberis* determined on a volume basis for sand and sawdust accentuates to a lesser degree the weight difference of the bedding materials and ensures a more appropriate comparison of number of *S uberis*. (*Am J Vet Res* 2001; 62:171–173)

Environmental mastitis is defined as those intramammary infections caused by pathogens whose primary reservoir is the environment in which the cows live.[1] Infectious agents are transferred from the environment to the cows, rather than being transmitted from infected mammary glands of other cows.[2] A primary reservoir for environmental bacteria is the bedding that cows lay on.[1]

Carroll[3] reported that bedding materials play a key role in the transfer of pathogens from the environment to the teats of cows as a result of the teats close contact with the bedding surface for extended periods. Populations of bacteria in bedding materials have been associated with an increase in contaminated teat ends by environmental bacteria and an increased incidence of intramammary infections.[4]

Researchers have investigated physical and chemical differences of bedding materials that make them more or less suitable environments for infectious pathogens.[5] Such information can be useful in making choices regarding bedding materials and improving bedding management to reduce bacterial exposure. Todhunter[6] stated that *Streptococcus uberis* is the most common species of environmental streptococci associated with mastitis in cows. Bramley[7] reported that $> 10^6$ **colony-forming units (CFU)** of *S uberis*/g of bedding material possibly resulted in an increased incidence of environmental mastitis.

Techniques for microbial culture of bedding materials have been used to evaluate the exposure potential of teats to bacteria that exist on the bedding surface.[2] Conventionally, bacterial counts in bedding have been reported on the basis of the number of CFU per gram of bedding material. However, this reporting method appears inappropriate when exposure potentials of various bedding materials are compared. For example, sand and sawdust are 2 common bedding materials that often are compared, but they have differing densities and probably possess differing exposure areas. The objective of the study reported here was to determine a method for comparing counts of *S uberis* in sand and sawdust that would be influenced to a lesser degree by densities of the bedding materials. A method that more clearly reflects exposure of teats to bacteria in the bedding would be useful for diagnostic investigations.

Materials and Methods

Bedding material—Bedding materials used in the study reported here included 2 sources of kiln-dried sawdust and 2 sources of washed sand. For each type of bedding, variation existed in texture and particle size. Bedding materials were measured on a volume basis by compressing bedding material in a metric-ruled cylinder, using a 1-kg drop weight. The 100 ml of packed bedding material was removed and weighed, using a metric scale. Measured bedding material was uniformly distributed to an approximate depth of 5 mm for sawdust and 3 mm for sand in a $30.5 \times 15.2 \times 7.6$-cm aluminum pan. Pans containing bedding were sealed with aluminum foil and steam sterilized at 150 C for 45 minutes.

Procedure—Two experiments were conducted. In both experiments, a culture of *S uberis* was used to inoculate sterilized bedding materials. Strains of *S uberis* were subcultured in 8 ml of Todd-Hewitt broth. After incubation at 37 C for 24 hours, strains were checked for purity by examination of gram-stained specimens. An 8-ml container of *S uberis* in Todd-Hewitt broth then was mixed with 228.8 ml of sterile saline (0.9% Na Cl) solution in a gas-sterilized spray bottle.

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Table 1—Least-squares means ± SEM of counts of *Streptococcus uberis* counts* in sand and sawdust bedding materials

	Experiment 1 (n = 19 replicates)		Experiment 2 (n = 16 replicates)	
Variable	**Sand**	**Sawdust**	**Sand**	**Sawdust**
Weight (g)	168.98 ± 3.50	31.60 ± 0.82	173.64 ± 2.59	22.47 ± 0.50
Volume (ml)	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Mean count*				
Volume basis	13.40 ± 0.05[a]	12.92 ± 0.05[b]	15.78 ± 0.08[a]	15.18 ± 0.08[b]
Weight basis	12.75 ± 0.05[a]	14.07 ± 0.05[b]	15.23 ± 0.08[a]	16.67 ± 0.08[b]
Mean difference*				
Volume basis	0.48 ± 0.05	−0.48 ± 0.05	0.60 ± 0.08	−0.60 ± 0.08
Weight basis	1.32 ± 0.05	−1.32 ± 0.05	1.44 ± 0.08	−1.44 ± 0.08

*Values are $\log_e$ number of colony-forming units.
[a,b]Within an experiment, values with different superscript letters differ significantly ($P < 0.001$).

For each replicate in each of the 2 experiments, a pan containing 100 ml of sterilized bedding was sprayed with 29.6 ml of *S uberis* inoculant. In experiment 1, bedding materials were inoculated with a mean (± SD) of $3.6 \times 10^6 \pm 4.2 \times 10^5$ CFU of *S uberis*/ml of bedding material. In experiment 2, bedding materials were inoculated with a mean (± SD) of $2.4 \times 10^7 \pm 3.1 \times 10^6$ CFU of *S uberis*/ml of bedding material. Five minutes after inoculation, 400 ml of saline solution was poured over the inoculated bedding, and the moistened bedding was agitated. Ten minutes after agitation, the wash solution was collected from the inoculated bedding, and 200-µl aliquots were removed. Each 200-µl aliquot was serially diluted 1:250 with 2 ml of saline solution and plated on tryptose agar with 5% sheep blood. A 200-ml aliquot of the serially diluted wash solution also was serially diluted with 2 ml of saline solution (final dilution, 1:2,500) and plated on tryptose agar with 5% sheep blood. Plates were incubated at 37 C for 48 hours. Plates containing 30 to 300 CFU of *S uberis*/ml of bedding material were used for calculating number of organisms, on the basis of dilution. The resulting number of *S uberis* per milliliter of bedding material was adjusted for the weight of bedding material to obtain the number of *S uberis* per gram of bedding material.

Data for plates contaminated with other bacterial species or when the count per plate was < 30 or > 300 CFU of *S uberis*/ml of bedding material were not included. However, none of the replicates were excluded for these reasons in either experiment.

The aforementioned procedure was replicated as many times as was allowed by the amount of bedding material that had been purchased. Consequently, experiment 1 consisted of 19 replicates for each bedding material, and experiment 2 consisted of 16 replicates for each bedding material.

Statistical analysis—Distributions of *S uberis* counts for sand and sawdust were normalized by use of $\log_e$ transformation. Statistical analysis was performed, using a commercial statistical program.[a] For each experiment, a general linear model ($\log_e$ CFU of *S uberis* = bedding material (ie, sawdust or sand) + basis (ie, volume or weight) + error) was used to detect differences in bacterial counts for each bedding material and each basis at the 95% confidence coefficient. Number of *S uberis* were separated and compared for each bedding material and each basis, using the Bonferroni method for multiple comparisons. Results were reported as Bonferroni **95% confidence intervals (95% CI)**. The Bonferroni method for multiple comparisons is a superior method when sample sizes are equal and the number of comparisons is equal to the number of factor levels, which was the case in this study (ie, bedding materials [sawdust or sand] and basis [volume or weight]). Reporting comparisons as Bonferroni 95% CI is a procedure that can be used to detect differences when *P*-values are similar.

Results

Analysis of results from experiments 1 and 2 revealed that $\log_e$ counts of *S uberis* calculated on a volume basis differed significantly ($P < 0.001$) from $\log_e$ counts of *S uberis* calculated on a weight basis (**Table 1**; **Fig 1 and 2**). The $\log_e$ counts of *S uberis* calculated on a volume basis for sawdust differed significantly ($P < 0.001$) from counts calculated on a volume basis for sand. Similarly, $\log_e$ counts of *S uberis* calculated on a weight basis for sawdust differed significantly ($P < 0.001$) from counts calculated on a weight basis for sand. Use of Bonferroni 95% CI intervals enabled us to distinguish significant differences.

Bedding materials in experiment 1 were inoculated with a mean of 3.6×10^6 CFU of *S uberis*/ml of bedding material. Resulting mean counts for *S uberis* cultured from wash solutions of inoculated sand and sawdust bedding were calculated (Table 1). The *S uberis* counts calculated on a volume basis for sand or sawdust differed significantly (Bonferroni 95% CI, -0.56 to -0.18). The *S uberis* counts calculated on a weight basis for sand or sawdust also differed significantly (Bonferroni 95% CI, 1.13 to 1.51). Reporting results in this manner reflected the difference between counts of *S uberis* for bedding material and basis of calculation as

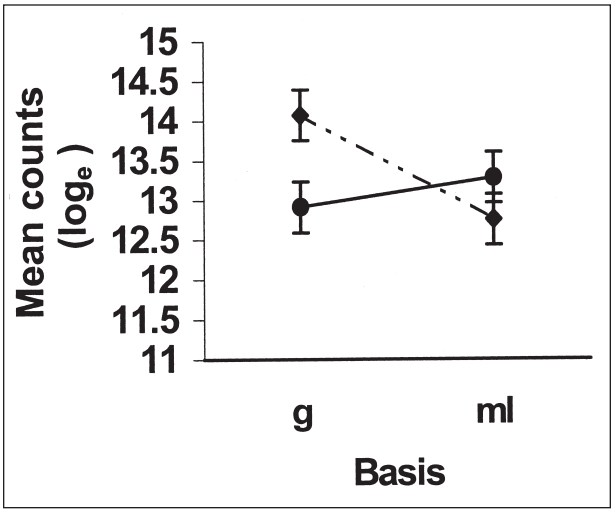

Figure 1—Graph of mean ± SD $\log_e$ count of *Streptococcus uberis* in sand (◆) and sawdust (●) bedding materials for experiment 1 on the basis of volume (ml) or weight (g) of bedding materials.

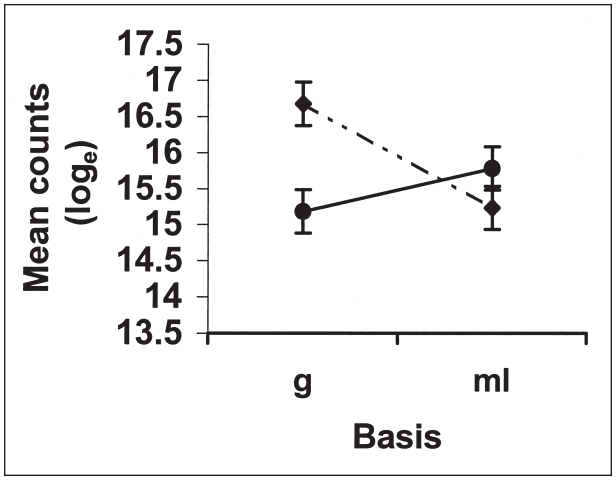

Figure 2—Graph of mean ± SD $\log_e$ count of *S uberis* in sand (u) and sawdust (●) bedding materials for experiment 2 on the basis of volume (ml) or weight (g) of bedding materials.

well as emphasizing the magnitude of the differences. The larger confidence intervals for counts of *S uberis* calculated on a weight basis for sand or sawdust documented the difference in density between sand and sawdust bedding materials.

Results for experiment 2 were similar to those for experiment 1. In experiment 2, bedding materials were inoculated with a mean of 2.4×10^7 CFU of *S uberis*/ml of bedding material. Mean counts for *S uberis* were determined (Table 1). Counts of *S uberis* calculated on a volume basis were significantly different between sand and sawdust (Bonferroni 95% CI, -0.89 to -0.31). Counts of *S uberis* calculated on a weight basis also were significantly different between sand and sawdust (Bonferroni 95% CI, 1.15 to 1.73).

Discussion

Studies on bacterial counts in bedding have been performed to provide information on quality of bedding materials. Traditionally, bacterial counts in bedding have been calculated on a weight basis.[1] Although this method may be appropriate for comparing bedding materials of similar density, it is not an appropriate means for comparing bedding materials of differing density. A volume-basis calculation allows investigators to compare bacterial counts for equal amounts of bedding material, which more closely reflects exposure area of bedding materials.

In the study reported here, analysis of results for 2 sources of sand and sawdust revealed that comparison on a weight basis is skewed in favor of the denser material. This point was suggested by the differences in the weight of 100 ml of each bedding material (Table 1). Bedding materials in each experiment were inoculated with the same number of *S uberis* colonies, and the same culturing technique was used for each repetition. Theoretically, if the bedding materials were the same in physical and chemical properties, the number of bacteria recovered should have been approximately the same for every replicate. Because the bedding materials differed in physical and chemical properties, we did not have identical recovery rates. Differences in absorb ency of the bedding materials could have accounted for some of the difference in recovery rates.[2]

In commercial dairy herds, bedding materials are applied to stall surfaces on a volume basis, not on a weight basis. For example, 1 shovel of sawdust may be adequate to cover the surface of a mattress in a free-stall facility. Volume more closely relates to the surface area that the bedding covers and, thus, is more representative of eventual exposure of the teats. Results from both experiments support the theory that counts of *S uberis* calculated on a volume basis accentuates to a lesser degree the difference in density between sand and sawdust bedding and more appropriately reflects the exposure area for *S uberis*. Higher mean counts of *S uberis* calculated on a weight basis for sawdust makes this apparent (Table 1). Large Bonferroni 95% CI values along with the large gap in plotted values (Fig 1 and 2) make it apparent that comparison of counts of *S uberis* calculated on a weight basis is not appropriate for comparing sand and sawdust.

Comparison of counts of *S uberis* calculated on a volume basis for sand and sawdust bedding accentuates to a lesser degree the difference in bedding density, ensuring a more appropriate comparison. Therefore, analysis of these data suggests that when bacterial counts of bedding materials are compared, the bacterial counts should be expressed on a volume basis (ie, CFU per milliliter of bedding material).

[a]MINITAB, version 12.2, Minitab Inc, State College, Pa.

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