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The impact of dairy cows' bedding material and its microbial content on the quality and safety of milk – A cross sectional study of UK farms

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ABSTRACT

The introduction of bedding dairy cows on recycled manure solids (RMS) in the UK led to concern by competent authorities that there could be an increased, unacceptable risk to animal and human health. A cross-sectional study was designed to evaluate the microbial content of different bedding materials, when used by dairy cows, and its impact on the microbial content of milk. Data were collected from farms bedding lactating cows on sand (n = 41), sawdust (n = 44) and RMS (n = 40). The mean duration of RMS use prior to sampling was 13 months. Total bacterial count, and counts of *Streptococcus/Enterococcus* spp., *Staphylococcus* spp., *Bacillus cereus*, thermophilic, thermoduric and psychrotrophic bacteria were determined in used bedding and milk. Samples were evaluated for the presence/absence of *Listeria monocytogenes*, *Salmonella* spp. and *Yersinia enterocolitica*. Data on milking practices were collected to investigate their potential to reduce microbial transfer from bedding to milk. There were substantial differences in bacterial counts both within and between bedding materials. However, there were no significant differences between bedding groups in counts in milk for any of the organisms studied, and no significant correlations between bacterial load in used bedding and milk. Fore-milking was associated with a reduced total bacterial count in milk. Dipping teats with disinfectant and drying, prior to milking, was associated with lower numbers of *Streptococcus/Enterococcus* spp. in milk. Disinfecting clusters between milking different cows was associated with a reduction in thermophilic and psychrotrophic counts in milk. This study did not provide evidence that use of RMS bedding increased the risk of presence of *Y. enterocolitica*, *Salmonella* spp. or *L. monocytogenes* in milk. However, the strength of this conclusion should be tempered by the relatively small number of farms on which *Y. enterocolitica* and *Salmonella* spp. were isolated. It is concluded that, despite the higher bacterial load of RMS, its use as bedding for lactating dairy cows need not be associated with a higher bacterial load in milk than the use of sand or sawdust. However, this finding must be interpreted in the light of the relatively recent introduction of RMS as a bedding material on the farms studied. Teat preparation provides a control point for the potential transfer of microorganisms from bedding to milk. The detection of zoonotic pathogens in a small proportion of milk samples, independent of bedding type, indicates that pasteurisation of milk prior to human consumption remains an important control measure.

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1. Introduction

The introduction of the use of recycled manure solids (RMS) as bedding for dairy cows in the European Union (EU) required that the relevant competent authorities be assured that there is "no unacceptable risk to animal and human health" (European Commission, 2009). The material described as RMS[1] is obtained by the physical removal of water from cattle slurry by pressure, aiming to attain a dry matter content of at least 34%. It has been used as cattle bedding for many years in the US (Keys et al., 1976; Timms, 2008) but only more recently in Europe, where it is largely used with no further processing (Feiken and van Laarhoven, 2012; Husfeldt et al., 2012; Marcher Holm and Pedersen, 2015). The material is known to carry a high bacterial load (Feiken and van Laarhoven, 2012; Harrison et al., 2008). Climatic conditions and dairy herd management differ between the US and the UK, and there is no peer reviewed published information on either the bacterial load of RMS as used in the UK, or the relationship between the use of RMS in UK conditions, and bacterial counts in milk. An initial scoping study identified knowledge gaps in the pathway of transfer of micro-organisms from bedding in general to milk under UK conditions (Bradley et al., 2014). Although some authors have reported associations between bacterial counts in bedding and milk (Magnusson et al., 2007; van Gastelen et al., 2011), such findings are not consistent. Bedding type *per se* was not found to be an explanatory factor for coliform and *Staphylococcus aureus* counts (Cicconi-Hogan et al., 2013) or total bacterial count in milk (Rowbotham and Ruegg, 2015). However, type of bedding material has been associated with counts of spore forming bacteria in milk in some studies[1] (Driehuis et al., 2012, 2014; Miller et al., 2015).

The bacterial load (numbers and types of organisms) of milk for human consumption has obvious implications for human health as well as for aspects of product quality, including suitability for certain types of processing, and keeping properties. Examples of organisms of concern to public health, which may be shed in bovine faeces with subsequent risk of milk contamination, are *Escherichia coli*, *Yersinia enterocolitica*, *Salmonella* spp. and *Listeria monocytogenes*.

Organisms such as *Bacillus cereus* (of concern in terms of food spoilage and potentially pathogenic), or *Clostridiaceae* such as *Clostridium tyrobutyricum* (of concern as a spoilage organism), are likely to be present in the farm environment (Julien et al., 2008; Vissers et al., 2007b) and may also contaminate milk (Bagge et al., 2005; Magnusson et al., 2007). *Bacillus* spp. and *C. tyrobutyricum* are among the bacteria which form spores with the ability to survive pasteurisation, and even ultra-heat-treatment, and are therefore also of concern with regard to keeping quality of milk (Gleeson et al., 2013; Miller et al., 2015). Among the *Bacillus* spp., *B. cereus* is the main concern from a food safety point of view (Lücking et al., 2013). Any of the aforementioned bacteria might be present in RMS, but also on any cattle bedding material which becomes contaminated with faeces. Mesophilic and thermophilic sporeformers are of particular concern for the quality of powdered milk (Sadiq et al., 2016). Although these organisms have previously been associated with composted bedding materials (Driehuis et al., 2012) the influence of other materials on their presence in milk is also worthy of investigation.

The objective of the current study was to evaluate the impact, on milk quality, of different materials, including RMS, when used as bedding for dairy cows under UK conditions. More specifically, the microbial contents of bedding and milk were evaluated, for the purpose of better understanding any potential risks to the quality and safety of milk produced for human consumption. Sawdust and sand were chosen as comparative materials for RMS, providing one organic and one inorganic comparison in a cross-sectional study of UK farms.

2. Materials and methods

2.1. Farm recruitment

The aim was to recruit a minimum of 40 farms into each of three groups, bedding at least 85% of the milking herd in freestalls on RMS, fresh sand (*i.e.* no farms recycled bedding sand, which is not a common practice in the UK) or sawdust respectively. Farms using RMS were recruited *via* contacts made during a previous scoping study (Bradley et al., 2014), distributors of separation machinery, veterinary surgeons and agricultural consultants. Sand and sawdust farms were identified through contacts of the research team, veterinary surgeons and participating farmers. Once the location of RMS farms was known, matching sand and sawdust farms were recruited, on the basis of approximate herd size (< 150 cows, 150–500 cows, > 500 cows) and geographical location within the UK (East/West). Matching on a North/South basis was not possible due to the limited number of farms using sand in the North of the UK. Milking method (parlour or automated milking system) was also used in matching. In the case of sand and sawdust farms, those carrying out regular recording of milk yields and individual cow somatic cell counts were preferentially selected; it was not possible to make this a requirement for RMS farms, due to their limited number in the UK.

2.2. Data collection and sample collection

Each farm was visited once by one of five experienced agricultural consultants, between 9th December 2014 and 31st March 2015. The consultants undertook prior training to ensure consistent methodology. Information on bedding management and milking practices was collected using a combination of a questionnaire and observations. Farm visits were arranged so that "used" bedding samples could be taken on a day when bedding was to be replenished, and these were collected just prior to the addition of fresh bedding, *i.e.* at the point of maximum contamination. At least 10 cubicles were sampled on each farm, proportionally distributed across different passageways and sheds using a previously agreed method. Approximately 75 mL of bedding material was collected from the top 2.5 cm layer from a standardised position at the rear of each cubicle, 15 cm in from the centre of the rear of the cubicle bed. If fresh faeces were present in the sampling position a new cubicle was selected. These samples were then comingled and thoroughly mixed. Sufficient cubicles were sampled to provide at least 750 mL of bedding. If there were two distinct types of bed design these were sampled separately; subsamples from the two types were later comingled proportionally according to the relative numbers of cubicles of the different designs. On the same day, a sample of 500 mL of thoroughly agitated milk was collected from the bulk tank ensuring that a full 24 or 48 h of milking had been accumulated and cooled. If more than one bulk tank was in use, both were sampled and subsamples were later comingled in proportion according to the relative volumes in the separate tanks. All samples were packed in insulated boxes with icepacks and immediately shipped to the laboratory for bacteriological analysis.

2.3. Laboratory methods

Samples of used bedding and milk were subjected to bacteriological analyses. Thirty grams of thoroughly mixed bedding material was added to 270 mL of maximum recovery diluent (MRD) and mixed in a stomacher for 1 min at 100 rpm prior to aliquoting for preparation of serial dilutions. Serial dilutions of milk and the bedding aliquots were

[1] RMS - recycled manure solids. [2] ToD - threshold of detection.

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then made in MRD to encompass the 2 or 3 dilutions anticipated to reflect likely counts. When necessary and where appropriate, further dilutions were undertaken to allow an accurate enumeration of colony forming units (CFU) to be determined. Growth was evaluated and enumerated on selective media 'pour plates', with the aim of allowing counts of a number of 'putative' bacterial populations to be made using recognised standard laboratory techniques (Campden BRI, 2007). The media used (all supplied by Oxoid Microbiology Products), the bacterial species enumerated and thresholds of detection (ToD) are outlined below:

Total Bacterial Count (TBC): Samples incubated in milk agar for 66–72 h at 30 °C (± 2 °C). (ToD ^{2}: Bedding: 10 CFU/g, Milk: 1 CFU/mL).
Coliform count: Samples incubated in VRB (MUG) agar for 66–72 h at 37 °C (± 2 °C). (ToD: Bedding: 10 CFU/g, Milk: 1 CFU/mL).
Laboratory Pasteurised Count (LPC): Samples heated to 63.5 °C (± 0.5 °C) for 35 min, then immediately cooled, prior to being incubated in milk agar for 66–72 h at 30 °C (± 2 °C). (ToD: Bedding: 10 CFU/g, Milk: 1 CFU/mL).
Streptococcus/Enterococcus spp. count: Samples incubated in Edwards agar for 66–72 h at 37 °C (± 2 °C). (ToD: Bedding: 10 CFU/g, Milk: 1 CFU/mL).
Staphylococcus spp. count: Samples incubated in Baird Parker agar for 48 h at 35 °C (± 2 °C). Colonies demonstrating morphology typical of *S. aureus* were then enumerated. (ToD: Bedding: 10 CFU/g, Milk: 1 CFU/mL).
Thermophilic Spore Count (TSC): Samples heated to 80 °C (± 1 °C) for 10 min then immediately cooled, prior to being incubated in milk agar for 24–48 h at 55 °C (± 2 °C). (ToD: Bedding: 10 CFU/g bedding, Milk: 1 CFU/mL).
Psychrotrophic count: Samples incubated in milk agar for 6 days at 5 °C (± 2 °C). (ToD: Bedding: 10 CFU/g bedding, Milk: 1 CFU/mL).
Bacillus cereus count: Samples heated to 80 °C (± 1 °C) for 10 min, then immediately cooled, prior to being incubated in "Brilliance" *Bacillus cereus* agar for 18–24 h at 35 °C (± 2 °C). Plates were re-examined after a further 18–24 h at room temperature. (ToD: Bedding: 10 CFU/g bedding, Milk: 1 CFU/mL).

In addition, specific enrichment and plating techniques to facilitate detection of additional pathogens of interest were undertaken as outlined below, with each test being carried out on duplicate aliquots of both bedding and milk.

Salmonella spp.: 25 g of bedding or 25 mL of milk was inoculated into 225 mL of Buffered Peptone Water (BPW) and incubated at 37 °C (± 2 °C) for 18–24 h. Following incubation, 100 μL of the BPW was inoculated into 10 mL of Rappaport-Vassiliadis (RV) enrichment broth and incubated at 42 °C (± 2 °C) for 24–48 h. Following this second incubation 10 μL of the RV broth was inoculated in duplicate onto Brilliant Green Agar and XLD Agar plates and incubated at 35 °C (± 2 °C) for 18–24 h. Suspicious colonies were identified by MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) (MALDI Biotyper, Bruker Daltonics). (ToD: Bedding: 1 CFU/25 g, Milk: 1 CFU/25 mL).
Listeria monocytogenes: 25 g of bedding or 25 mL of milk was inoculated into 225 mL of *Listeria* Enrichment Broth (LEB) and incubated at 30 °C (± 2 °C) for 7 days. LEBs were then sub-cultured at 1, 2 and 7 days onto *Listeria* Selective Agar (LSA) and incubated at 35 °C (± 2 °C) for up to 48 h. Suspicious colonies were identified by MALDI-TOF MS. (ToD: Bedding: 1 CFU/25 g, Milk: 1 CFU/25 mL).
Yersinia enterocolitica: Both a 100 μL and a 10 μL aliquot of the 10^{-1} dilution of milk or bedding were inoculated on *Yersinia* selective agar and incubated for 18–24 h at 32 °C (± 2 °C). Suspicious colonies were identified by MALDI-TOF MS. (ToD: Bedding: 100 CFU/g, Milk: 100 CFU/mL).

All bacterial counts from bedding were expressed per gram fresh weight of bedding material, and per millilitre of milk.

Dry matter (DM) content of used bedding was determined. Two subsamples of 50 g sand, 20 g sawdust or 20 g RMS were used for determination of DM content, by drying to constant weight in an oven.

2.4. Data collation and statistical analysis

Data were collated and initially analysed using Excel and Access 2003 (Microsoft Corp) and Minitab 15.1 (Minitab Inc.). Herd size and milk sales per cow were compared between the three groups using ANOVA, followed by a two-sample *t*-test for pairwise comparisons, to test for consistency between groups. The proportion of farms employing particular practices was compared using the Chi-squared test. Bacterial counts in bedding and milk for the three groups were compared using the Kruskal-Wallis test because data were neither normally distributed, nor normalised by log transformation. For some organisms this was influenced by outliers, and for others by a large number of samples in which the organism was not detected. Individual pairwise comparisons were made using the Mann-Whitney *U* test. For *Salmonella* spp., *L. monocytogenes* and *Y. enterocolitica*, the proportion of samples testing positive in each group was compared using the Chi-Square test, using a Yates correction for small numbers where required. A layered Bonferroni correction was used to allow for multiple comparisons where appropriate (Darlington, 1990). Relationships between individual bacterial counts in bedding and milk on the same farm were tested using correlation, across all bedding types.

To investigate the influence of milking practices, the Kruskal-Wallis test was used to compare bacterial counts in milk, across all bedding types, for farms which did or did not employ particular management practices: foremilking; predip followed by dry wipe; any form of cluster disinfection between milking different cows (whether automatic or manual, between some cows or all cows) and a hot wash of the milking plant after every milking.

3. Results

3.1. Farm descriptors

The three groups did not differ significantly in herd size; mean herd size was 374 (sd 217) for RMS, 370 (sd 248) for sand and 336 (sd 191) for sawdust. Mean annual milk sales per cow were significantly higher for sand farms (9446 L, sd 1367) than for RMS farms (8803 L, sd 1140, $P = 0.048$) or sawdust farms (8491 L, sd 1090, $P = 0.003$). Key farm and management descriptors are presented in Table 1. Key descriptors relating to the design and management of beds are summarised in Table 2. Sawdust was used, with one exception, as a thin layer on mats or mattresses, as is typical in the UK. No distinction has been made between mats and mattresses in this study. Sand was predominantly used in "deep" beds (at least 4 cm depth of bedding, in an enclosed structure). RMS was used both in deep beds ($n = 11$) and on mattresses ($n = 20$); nine RMS farms had some beds of each type. The mean depth of deep beds did not differ significantly between sand and RMS beds (14.8 cm (sd = 5.70) and 14.6 cm (sd = 6.67) respectively).

Typically, sawdust was replenished once or twice daily, RMS daily or every other day, and sand once or twice per week (always with fresh rather than recycled sand). Seventy-eight percent of sawdust beds were replenished at least once a day, while 99% of sand beds were replenished less frequently than daily, and 39% weekly or less often. Forty-two percent of RMS beds were replenished daily, and 42% every

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Table 1
Summary of key farm descriptors of study farms.

Variable	Bedding	n	Mean	Median	SD	Min	Max	25th percentile	75th percentile
Average herd size									
	RMS	40	374	290	217.0	135	1000	220	480
	Sand	41	370	300	248.7	120	1550	228	435
	Sawdust	44	336	265	191.7	110	1020	205	425
Milk sales L/cow/year									
	RMS	40	8803[a]	8663	1140	6500	10,833	7895	9766
	Sand	41	9446[b]	9524	1367	6567	12,115	8473	10,419
	Sawdust	43	8491[a]	8333	1090	5902	10,435	7800	9308
Stocking rate (cows/100/cubicles) on day of visit									
	RMS	40	96.1	97.7	9.70	69.4	116.4	92	101
	Sand	41	99.3	98.4	11.51	75.3	137.2	94	104
	Sawdust	44	94.4	95.3	7.86	72.9	111.4	91	99

[a,b]Values with different superscripts within columns, within parameters, differ (RMS v Sand P = 0.048, Sawdust v Sand P = 0.003).

Table 2
Summary of types of bed and bedding management for different bedding materials on 125 study farms.

	RMS	Sand	Sawdust	Total
Bed structure				
All mats or mattresses with a covering of bedding material	20	1	42	63
All "deep" beds[a]	11	36	1	48
Mixed types	9	4	1	14
Frequency of replenishing bedding				
More than once a day	0	0	21	21
Daily	16	1	13	30
Less often than daily but more often than weekly	24	23	10	57
Weekly or less often	0	17	0	17
Frequency of scraping manure off beds - times per day				
1	1	0	0	1
2	24	26	39	89
3	14	15	5	34
4	1	0	0	1
Frequency of moving bedding from front to back of cubicles – times per day				
0	4	6	7	17
1	3	2	2	7
2	21	23	32	76
3	11	10	3	24
4	1	0	0	1
Frequency of regular raking of bedding				
Daily or more often	10	16	3	29
Less often than daily	2	1	0	3
No regular raking	28	24	41	93

[a] *i.e.* enclosed structure containing bedding material with at least 4 cm depth.

other day. Management of sand beds consisted of removing fresh faeces two or three times per day, and levelling beds with variable frequency, usually by hand, occasionally with machinery (Table 2). No farms carried out mechanical "cultivation" of sand. Beds of sawdust and RMS were usually cleaned manually (faeces removed) at every milking, and often bedding from the front of the cubicle would be pulled towards the back at this time.

Milking frequency, cluster disinfection and teat preparation practices on the farms are summarised in Table 3. Twice daily milking was more common on sawdust farms than in the other two groups. A minority of farms used automatic milking system for at least part of the herd. Automatic cluster disinfection was used on 47.5% of the RMS farms, 46.3% of sand farms and 32% of sawdust farms. Sixty-five per-

Table 3
Summary of milking practices on study farms.

Parameter	RMS	Sand	Sawdust	All
n	40	41	44	125
Milking frequency				
× 2	22	24	37	83
× 3	13	14	3	30
Part of herd × 3	0	1	2	3
All AMS	2	2	2	6
Some AMS	3	0	0	3
Cluster disinfection				
Automatic	19	19	14	52
Manual after all cows	0	1	0	1
Manual after some cows	11	14	24	49
Mixed	1	0	0	1
None	9	7	6	22
Teat preparation				
Dry wipe	4	5	7	16
Medicated wipe	0	3	4	7
Predip	26	28	22	76
Brush (including on AMS)	8	5	5	18
AMS wash and dry	1	0	0	1
Wash only	0	0	1	1
Unknown	1	0	5	6
Hot plant wash				
After every milking	19	27	25	71
Not after every milking	21	14	19	54

AMS = automated milking system (robot).

cent of RMS farms, 68% of sand farms and 50% of sawdust farms used a pre-dip. A hot plant wash was used after every milking on 47.5% of RMS farms, 65.9% of sand farms and 56.8% of sawdust farms. These proportions were not significantly different.

The mean duration of RMS use by the time of the visit was 13.6 months (median 13.6, range 1–35 months). All farms using sand and sawdust bedding had been doing so for at least 24 months.

3.2. Dry matter content of used bedding

Mean DM contents of used bedding materials were: RMS 44.5% (sd 7.29), sand 94.9% (sd 1.90) and sawdust 76.2% (sd 7.57).

3.3. Bacterial counts in used bedding

Bacterial counts on used bedding samples are summarised in Fig. 1. For the majority of species and groups enumerated, bacterial counts in bedding were significantly higher in RMS than either sand or sawdust. Numerically, mean and median counts were typically lowest on sand farms. The exception was the *Streptococcus/Enterococcus* spp. for which

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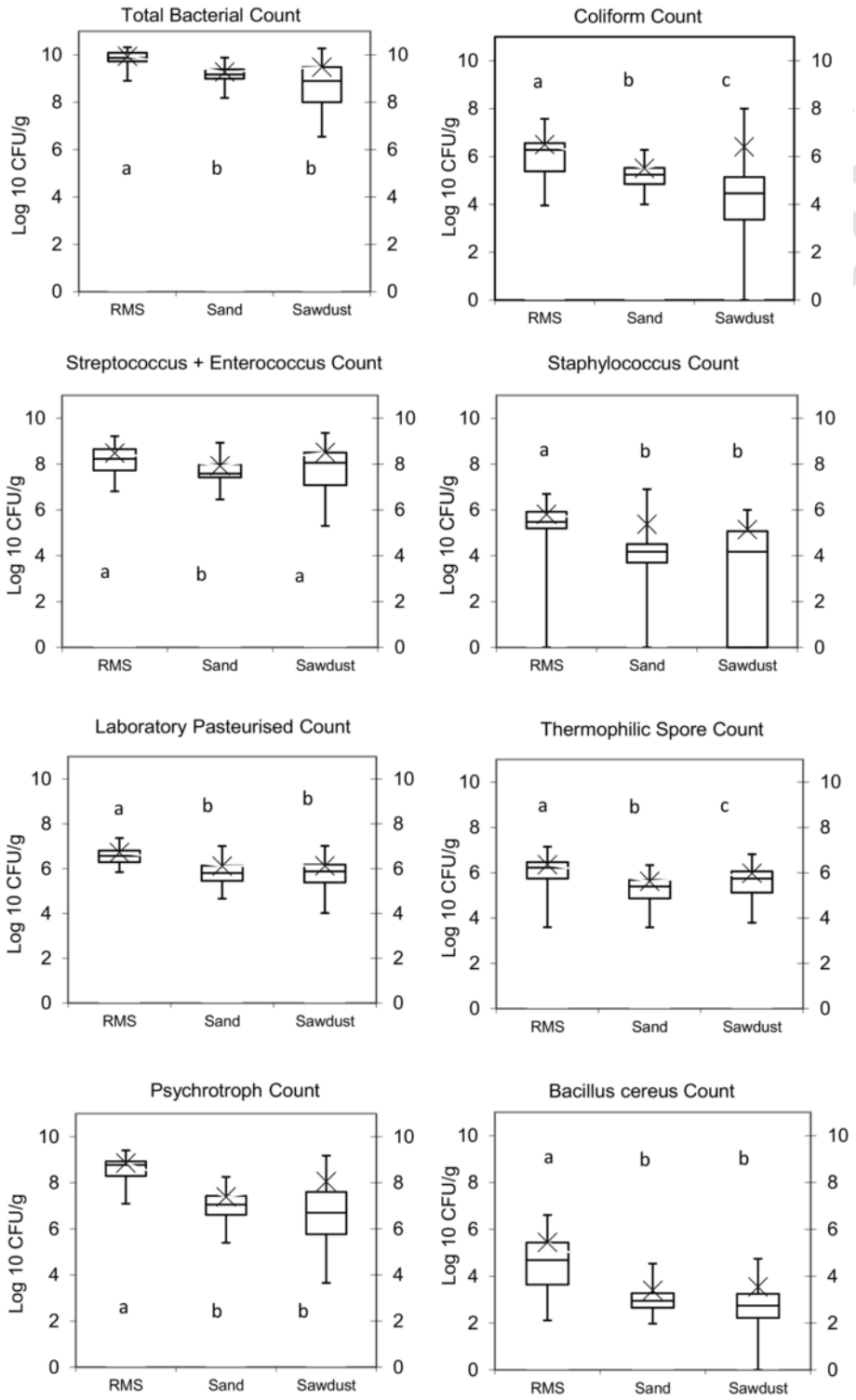

Fig. 1. Bacterial counts (log_{10} CFU/g) in used bedding material for milking cows from farms using recycled manure solids (RMS) (n = 40), sand (n = 41) and sawdust (n = 44). Box plots illustrate the median, quartiles, and minimum and maximum values (as bars). Logs of arithmetic means are indicated by crosses. Different letters between different groups indicate a significant difference according to Kruskal-Wallis with Mann-Whitney used for individual pairwise comparisons and Bonferroni correction for multiple comparisons. $P < 0.001$ with two exceptions: Thermophilic Spore Count RMS v sawdust, $P < 0.01$, Thermophilic Spore Count sand v sawdust, $P < 0.05$.

counts in RMS did not differ from those in sawdust, both being significantly higher than counts in sand. However, there was often as much variation within as between bedding types.

TBCs in bedding were not significantly different between sand and sawdust farms. Only one RMS farm had a TBC in the bottom quartile of all those measured while only one sand farm was in the upper quartile. While 50% (22/44) of the sawdust farms were in the lowest quartile of TBCs, 18% were to be found in the upper quartile.

Coliform counts in bedding were lowest on sawdust farms with the median count (2.9×10^4 CFU/g) being one log_{10} less than on sand

farms (1.8×10^5 CFU/g) (P = 0.0004) and 2 $logs_{10}$ less than on RMS farms (1.9×10^6 CFU/g) (P < 0.0001). However, twenty-five of the lowest 30 coliform counts were recorded on sand farms and the highest coliform count was reported on a sawdust farm (1.0×10^8 CFU/g).

Streptococcus/Enterococcus spp. counts were lower on sand farms than either sawdust or RMS farms (P = 0.0003) but were not significantly different between sawdust and RMS farms; again there was a large amount of variation within groups. The highest (2.3×10^9 CFU/g) and lowest count (2.0×10^5 CFU/g) were reported on sawdust farms. RMS farms were more evenly distributed on *Streptococcus/Enterococcus* spp. count than for other counts.

Staphylococcus spp. counts varied dramatically between farms, within each bedding material, ranging overall between < ToD (< 10 CFU/g) and 8×10^6 CFU/g. Two thirds of the upper quartile comprised RMS farms, although the highest count was recorded on a sand farm. Counts were significantly higher in RMS than either sand (P = 0.0003) or sawdust (P = 0.0003).

LPCs were highest on RMS farms (P < 0.0001) with no RMS farms being represented in the lowest third of counts. Sand and sawdust farms did not differ and were evenly distributed, with < 10% appearing in the upper quartile of counts.

TSCs were significantly different between all bedding types, being lowest on sand farms and highest on RMS farms (RMS v sand P < 0.0001, RMS v sawdust P = 0.003, sand v sawdust P = 0.0267). Half the RMS farms were in the upper quartile of all farms; however, three of the lowest six counts were recorded on RMS farms.

Bacillus cereus was detected (threshold = 10 CFU/g) in 70% of RMS samples, 5% of sand samples and 11% of sawdust samples. *B. cereus* counts were significantly higher in RMS beds (P < 0.0001), being 2 $logs_{10}$ higher than on either sand or sawdust farms, which did not differ from each other. On one RMS farm, in excess of 4×10^6 organisms were present per gram of bedding.

No effect of duration of use of RMS on bacterial counts in bedding was detected.

3.4. Presence/absence of specific zoonotic organisms in bedding

Y. enterocolitica was identified in the bedding on 4.9% of sand farms, 5% of RMS farms and 9.8% of sawdust farms, but the prevalence did not vary significantly between bedding types (Table 4). *Salmonella* spp. was identified in used bedding on four farms (two sand and two RMS) (Table 4). No isolations were made from bedding collected during the winter months (*i.e.* before 9th March). The isolate from both RMS farms and one sand farm was identified as *Salmonella enterica* serovar Mbandaka, the isolate from the second sand farm was identified as *Salmonella enterica* serovar Montevideo. Freshly generated RMS was subsequently sampled on one RMS farm 22 days and 82 days later and on the other 70 days later - on all three occasions the same *Salmonella* spp. were re-isolated from the freshly separated solids. *L. monocytogenes* was isolated from bedding on a significantly higher proportion of sand farms (58.5%) than RMS (15.0%) (P < 0.0001) or sawdust farms (31.7%) (P = 0.0071), which did not differ (Table 4).

3.5. Bacterial counts in milk

Results of bacterial counts from milk are presented in Fig. 2. Across all the species and groups enumerated bacterial counts in bulk milk did not differ across the farms bedding on different materials. *B. cereus* was only identified in milk on five farms: three RMS, one sand and one sawdust. In all cases there were only 5 or 10 CFU/mL of milk.

3.6. Presence/absence of specific zoonotic pathogens in milk

Y. enterocolitica was identified in the bulk milk of 0%, 5% and 12.2% of sawdust, RMS and sand farms respectively, but the prevalence did not vary significantly between bedding types. A *Salmonella* spp. was identified in the bulk milk of one sawdust farm and was subsequently identified as *S.* Montevideo (Animal and Plant Health Agency). *L. monocytogenes* was isolated from bulk milk from 2.4% of sand farms, 12.2% of sawdust farms, and 12.5% of RMS farms; however, the prevalence did not vary significantly between bedding types (Table 5).

3.7. Relationships between bacteria in bedding and milk

No significant correlations were identified between bacterial numbers in bedding and in bulk milk for any of the bacterial groups enumerated. No effect of duration of RMS use upon bacterial counts in milk was demonstrated.

3.8. Influence of milking practices on bacterial counts in milk

Across all bedding types, fore-milking was associated with a lower TBC in bulk milk (median: 2503 *vs* 4800 CFU/mL; P = 0.047), but not with any other bacterial species/grouping. Pre-milking teat preparation that involved a pre-dip followed by wiping dry was associated with a lower *Streptococcus/Enterococcus* spp. count in bulk milk (median: 340 *vs* 650 CFU/mL; P = 0.023), but not with difference in any other bacterial species/grouping. Disinfecting clusters between milking different cows was not found to be associated with lower bacterial counts in milk, with the exception of TSCs and psychrotrophic counts. TSCs were significantly lower in the bulk milk of farms employing any form of cluster disinfection (whether automatic or manual, between some cows or all cows) than those not employing any cluster disinfection (median: 35 *vs* 62.5 CFU/mL; P = 0.01). Similarly, psychrotrophic counts were significantly lower in the bulk milk of farms employing any form of cluster disinfection than those not employing cluster disinfection (median: 125 *vs* 245 CFU/mL; P = 0.04). No difference was detected between manual (n = 50) and automated (n = 52) cluster disinfection systems. There was a trend for a hot wash after every milking to be associated with a reduction in the laboratory pasteurised count in milk (median: 272.5 *vs* 190 CFU/mL; P = 0.144), but not with any other bacterial species/grouping.

Table 4
A summary of the proportion of farms from which *Yersinia enterocolitica*, *Salmonella* spp. and *Listeria monocytogenes* respectively were isolated from bedding (values within rows with different superscripts differ P < 0.01).

Organism	RMS (n = 40)		Sand (n = 41)		Sawdust (n = 44)	
	n	%	n	%	n	%
Y. enterocolitica	2	5.0	2	4.9	4	9.8
Salmonella spp.	2	5.0	2	4.9	0	0.0
L. monocytogenes	6	15.0[a]	24	58.5[b]	13	31.7[a]

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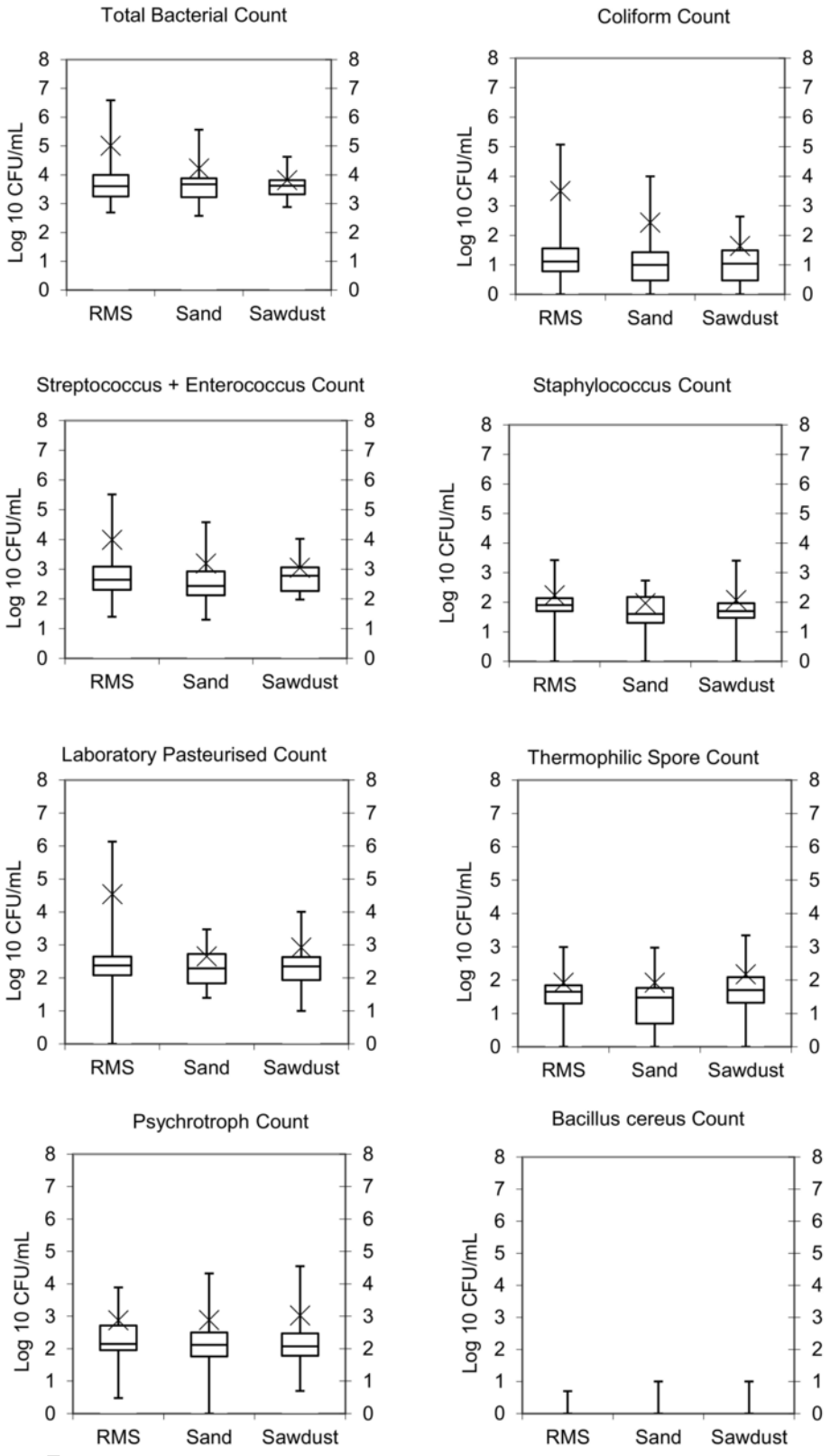

Fig. 2. Bacterial counts (log_{10} CFU/mL) in bulk milk from farms using three different bedding materials for milking cows. Box plots illustrate the median, quartiles, and minimum and maximum values (as bars) for farms using recycled manure solids (RMS) (n = 40), sand (n = 41) and sawdust (n = 44). Logs of arithmetic means are indicated by crosses. No significant differences between groups were demonstrated, using the Kruskal-Wallis test.

Table 5
A summary of the proportion of farms from which *Yersinia enterocolitica*, *Salmonella* spp. and *Listeria monocytogenes* respectively were isolated from bulk milk.

Organism	RMS (n = 40)		Sand (n = 41)		Sawdust (n = 44)	
	n	%	n	%	n	%
Y. enterocolitica	2	5.0	5	12.2	0	0.0
Salmonella spp.	0	0.0	0	0.0	1	2.4
L. monocytogenes	5	12.5	1	2.4	5	12.2

4. Discussion

The ultimate aim of the study was to determine whether bedding type influenced milk quality, when a comparison was made between sand, sawdust and RMS as currently used under UK conditions. The study provided an overview of bacterial populations in used bedding materials, under existing UK management regimes. Necessarily in an observational survey, the comparisons made were of the combined effects of bedding material and bedding management on each farm, and must be interpreted as such. There were wide ranges in counts for all bacterial groups, both within and between bedding types. Overall, as might be expected from the nature of the raw materials, there were significantly higher counts in RMS compared with sand and sawdust. However, the high counts of some individual sand and sawdust samples is worthy of note as it illustrates that very high bacterial counts can still occur in these materials, which are often considered "clean", particularly sand. The highest count for *Streptococcus/Enterococcus* spp. was found in sawdust and for *Staphylococcus* spp. in sand. The differences in the counts in bedding were not transferred to milk, so the hypothesis that type of bedding would affect microbial counts and flora in milk was not supported. Neither was there any correlation between counts in bedding and in milk, across the three bedding materials. Of the three individual zoonotic bacteria analysed for presence and absence (*Salmonella* spp., *L. monocytogenes* and *Y. enterocolitica*), *L. monocytogenes* were most frequently isolated overall, and most commonly found in sand. This could have been related to the source of the sand (and soil contamination) or have been a feature of the fact that sand was replenished less frequently, allowing a build-up of *L. monocytogenes* of faecal origin. No pattern or association of presence of a particular zoonotic organism in milk with a particular bedding type was seen. The prevalence of detection of these bacteria in milk was low, so this comparison needs to be interpreted with care. The isolation of these organisms from bedding and milk is a reminder of the potential risk to human health presented by the farm environment and consumption of unpasteurised milk. The ability to influence the transfer of bacteria from bedding to milk by teat preparation was demonstrated by the association of pre-dipping with a lower *Streptococcus/Enterococcus* spp. count in bulk milk.

The findings of the study are limited by the relatively small population of farms using RMS, and the fact that they were necessarily a self-selecting population of successful users of this new technology. The recent introduction of the practice restricted the length of time the material had been in use. When one considers the direct physical transfer of organisms from bedding to milk, it would be reasonable to expect any changes associated with a bedding material to occur almost immediately. However, aspects such as the absolute levels of bacterial numbers in bedding, and the balance between species, could potentially alter with time or with climatic conditions. It should be noted that the study was restricted to the winter and spring. The study did not provide information on the diversity or balance of species within the broad groups identified by the selective media used. Neither did the study reveal genetic characteristics of the bacteria, including antimicrobial resistance, which might also change over time. Notwithstanding the comments above, our analysis did not reveal any evidence of a relationship between the duration of use of RMS and bacterial numbers in bedding or milk.

Comparisons of bacterial load in bedding were made on a fresh weight basis to allow direct comparison with the most recent publications on this subject (Driehuis et al., 2012, 2013, 2014; Rowbotham and Ruegg, 2015, 2016; Sorter et al., 2014). Some have argued that presentation on a volume basis has greater biological significance (*e.g.* Harrison et al., 2008), but we considered that the transfer of micro-organisms from bedding to teats may also be related to other physical parameters influencing adherence to the teats, so counts made on a volume basis may not be directly related to the risk of transfer either. Determining the optimum or minimum management regime for each material was beyond the scope of this study.

Considering the bacterial load of the used bedding, it is perhaps surprising that the TBCs were not significantly different between used sand and sawdust, being an inorganic and an organic material, but several authors have reported how rapidly bedding materials become contaminated once in contact with cows (*e.g.* Cole and Hogan, 2016; Harrison et al., 2008). Also, the difference in bedding management styles for different materials needs to be considered. RMS was most typically replenished either daily or every other day, sawdust at least once daily, but often at each milking, and sand once or twice per week. Sawdust as a material might not have maintained such low counts if bedded less frequently, while sand beds were at least as "clean" as sawdust (with the exception of the coliform count), despite being bedded much less frequently. This supports the industry perception that sand, as an inorganic bedding material, is less conducive to bacterial growth than organic materials. Comparisons of different management methods did not fall within the scope of this study, and the study does not demonstrate how RMS or sawdust would perform if replenished less frequently.

The median coliform levels in RMS beds in the present study were similar to the means reported in a recent controlled study from Wisconsin for RMS from deep beds, but two $\log_{10}$ higher than that study's treatment group with shallow beds of RMS (Rowbotham and Ruegg, 2016). For sand beds, the Wisconsin mean coliform count was below our minimum count. There are a number of possible contributing factors to this difference. First, Rowbotham and Ruegg (2016) froze bedding samples prior to analysis, which has been shown to reduce the recovery of Gram negative organisms (Homerosky and Hogan, 2015). Secondly, Rowbotham and Ruegg (2016) sampled the top 8 cm of bedding material, rather than the top 2.5 cm, with the possible outcome that surface faecal contamination would be diluted by cleaner bedding from the deeper layers, at least in deep beds. Thirdly, in the American study, sand beds were replenished twice weekly, while in this survey, 54% of herds had fresh sand added less often. Levels of *Streptococcus/Enterococcus* spp. were closer in the two studies, although counts in sand were still 0.64 $\log_{10}$ lower in the American samples. It is also highly likely that differing climatic conditions of the two studies may have had an influence on bacterial counts, which is why it was considered important to collect UK data. The median levels of *B. cereus* spores detected in "used" sawdust and sand in this study (2.7 and 3.0 $\log_{10}$ respectively) were similar in order of magnitude to the means found in manure solids, straw and sawdust from cubicles by Driehuis et al. (2013), which ranged from 2.3 $\log_{10}$ to 2.6 $\log_{10}$. The median of 4.7 $\log_{10}$ for used RMS in the current study was somewhat higher. Driehuis et al. (2013) enumerated spores of mesophilic aerobic spore-forming bacteria in samples of bedding and milk, after pasteurisation of 10 min at 80 °C, as in the current study. Their protocol could have cultured a

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slightly different population of organisms because incubation was 48 h at 37 °C compared with 24–48 h at 55 °C for the "thermophilic spore count" in the present study. Despite this possible discrepancy, in the two studies the spore counts were of similar order in RMS, and significantly lower in the "control materials".

The lack of influence of type of bedding on bulk milk bacterial counts is in agreement with the findings of a previous epidemiological analysis modelling data from 325 US farms where bedding types were categorised as inorganic, manure based, or organic (Rowbotham and Ruegg, 2015). There, bulk milk TBC, measured monthly over a two-year period, was not found to be related to type of bedding, or any aspect of management of the bedding. Miller et al. (2015) found no difference in TSC in milk between farms bedding with sand, sawdust and recycled manure, although straw bedding decreased the odds of TSC exceeding 10/mL in milk. Similarly, Driehuis et al. (2013) did not discover any differences in *B. cereus* counts of bulk tank milk from Dutch herds with straw, sawdust or RMS bedded cubicles. van Gastelen et al. (2011) reported a weak correlation between TBC in bedding and milk on 16 farms with a variety of bedding materials. Magnusson et al. (2007) found a positive correlation ($r = 0.75$, $P < 0.001$) between *B. cereus* spore counts in bedding and milk, sampled on seven farms where bedding materials included straw, sawdust and sand, with evidence of transfer from sawdust to milk on the one farm where this was studied using RAPD-PCR. This is of note, given the importance of *B. cereus* as a potential food poisoning organism. In the present study, the lack of correlation between *B. cereus* counts in bedding and milk is likely to have been dominated by the very low prevalence of *B. cereus* in milk (only detected in 4% of milk samples). A much higher prevalence and level of milk contamination were reported in an Irish study made while cows were grazing (O'Connell et al., 2013). Magnusson et al. (2007) reported that when concentration of spores in bedding exceeded 10,000/g (4 $\log_{10}$/g) there was increased risk of levels in milk exceeding 100/L (2 $\log_{10}$/L). Their counts based on a filtration method permitted a lower detection threshold than in the present study, and thus the establishment of such a relationship. In the present study, the detection threshold of 1 CFU/mL (*i.e.* 1000 CFU/L) did not allow comparison with this finding to be made.

It is possible that management of bedding introduces more variation of bacterial counts in milk than does bedding type *per se*. The fact that certain management methods were commonly applied with certain bedding materials prevented investigating this in the present study. Physical attributes of bedding material which may or may not be related to the type of material *per se* will influence properties such as adherence to the teats. For example, the greater differential between bacterial load in bedding and in milk when RMS is used, might arise if RMS is less liable to stick to the teats than sand or sawdust. Vissers et al. (2007a) estimated that there was a 100-fold difference between farms in the amount of dirt transferred from cows to the bulk tank. Part of this difference may be due to pre-milking teat preparation methods (Elmoslemany et al., 2010), influencing both physical contamination of milk, and intramammary infection. Our study demonstrated a reduction in *Streptococcus/Enterococcus* spp. in bulk milk associated with pre-milking teat disinfection followed by a dry wipe, regardless of the bedding type. However, the effect of pre-dipping reducing milk TBC (Piepers et al., 2014), and psychrotrophic counts (Elmoslemany et al., 2010) was not demonstrated in our study. There may be interactions between teat preparation methods and type and management of bedding which were too complex to be revealed by this study.

Specific pathogens of zoonotic concern selected for investigation in this study were *B. cereus*, *L. monocytogenes*, *Salmonella* spp. and *Y. enterocolitica*. Detection of *B. cereus* in 4% of milk samples is comparable with a report that 3.8% of all milk samples tested positive for *B. cereus* in an EU survey (EFSA, 2012). Milk from the farms with positive tests in this study is within the EFSA data range of < 2 $\log_{10}$ to 4.3 $\log_{10}$ spores/L. However, the positive samples would not satisfy the requirement for raw milk for processing, of being below 3 $\log_{10}$ spores/L, given by Walstra et al. (2005).

Finding the highest prevalence of *L. monocytogenes* in sand bedding was perhaps surprising. However, as *Listeria* spp. are soil borne organisms, and some of the sand was sourced from pits and quarries, it is possible that the prevalence of *L. monocytogenes* was influenced by the source. The extent of information on sand sources, and the number of samples from different types of source, were insufficient to demonstrate any relationship with the source of sand. The overall prevalence of *L. monocytogenes* in milk samples at 8.8% was relatively high compared with a recent report from Italy of 2.2% in 5897 raw bulk tank milk samples collected over four years (Dalzini et al., 2016), but within the range of 1.3 to 12.6% indicated in an international review by Oliver et al. (2005). The Italian study noted that the prevalence was highest in Spring and Autumn, while a French study (Meyer-Broseta et al., 2003) found a peak in winter, possibly attributable to feeding silage, while the Italian herds were fed hay in winter. Samples in the present study were all collected during the winter housing period, but with no obvious time-related pattern in prevalence of positive samples over the course of the study.

The prevalence of 0.8% of *Salmonella* spp. in milk samples was at the low end of the range reported in the review by Oliver et al. (2005) (0.2 to 8.9% isolation rate), though it is worthy of note that the isolation of *Salmonella* spp. on the two farms using RMS was associated with isolation of the same *Salmonella* spp. at a later date. This could reflect long-term recycling of this pathogen, but equally could also indicate an ongoing contribution from an environmental source (*e.g.* wildlife). *Salmonella* positive samples were all collected during the latter part of the study, which may have been a chance finding, but could have reflected a time when environmental conditions were more favourable to its survival.

Comparative data on isolation of *Y. enterocolitica* is more limited. Ruusunen et al. (2013) detected *Y. enterocolitica* in 7.7% of Finnish bulk milk samples, but considered that the isolates were non-pathogenic (negative for the *ail* gene). The overall isolation rate was slightly lower in the present study at 5.6%, but information on the pathogenicity is not available.

This study gave no evidence of an influence of use of any of the three bedding materials studied upon the levels of heat resistant spores in milk. This is as might be expected since none of the materials had undergone a composting process, which was the common feature of the materials found by Driehuis et al. (2012) to be associated with elevated levels of spores of mesophilic and thermophilic spore-formers in milk. This, in combination with the lack of influence of bedding type on psychrotrophic counts in milk, means that no evidence was found for use of any of these three materials as bedding being particularly detrimental to the keeping qualities of milk, or heat-treated milk products.

This study is the first peer reviewed report of the influence of RMS use on milk quality in European conditions, although considerable work on the influence of bedding materials on aerobic spore-formers in bedding and milk has been carried out in the Netherlands (Driehuis et al., 2012, 2013, 2014). As such, it provides data that is more relevant to Europe than previous reports from the US. The study population of RMS farms is considered to be a good representation of those operating in the UK, with the proviso already given, that farms using RMS were unavoidably limited by the recent adoption of the technique, and represent those which have been initially successful in adoption of this technology. At the time of the study, it was not necessary for UK farmers to register their use of RMS, but we believe that over 50% of the farms using RMS were included. Overall, the farms in the study included 0.9% of all UK dairy holdings and 2.4% of the national dairy cow population for 2015 (AHDB). Average herd size for the RMS group was larger than the UK average (374 v 143 cows) and annual yield per

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cow was higher than the national average (8803 v 7912 L). Sand and sawdust farms were recruited to match the RMS farms, thus the overall study population also exceeded the national average in terms of herd size and milk yield per cow (overall study means: 359 cows yielding 8907 L/year). The study was carried out in the winter and spring, corresponding to the traditional housing period for UK herds; if cows are housed during the summer, in warmer conditions, bacterial populations may be different.

It is concluded that, despite the higher bacterial load of RMS, its use as bedding for lactating dairy cows need not be associated with a higher bacterial load in milk, when compared to the use of sand or sawdust. However, RMS, and for some bacterial groups, other bedding materials, have the potential to transfer large numbers of bacteria to the teats. Milking practices, particularly teat preparation, provide a vital critical control point for reducing transfer of bacteria to milk for consumption. The detection of zoonotic pathogens in a small proportion of milk samples indicates that pasteurisation of milk prior to human consumption remains an important aspect of ensuring the safety of milk as a food product.

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