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Biochar supplementation affects the microbiome of recycled manure solids for cow bedding: a metagenomic analysis

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ABSTRACT

The widespread use of Recycled Manure Solids (RMS) as cow bedding material is not without risks, since cattle manure may act as a vehicle for pathogenic and antimicrobial resistant bacteria dissemination. Thus, our aim was to evaluate RMS-supplemented with a pine biochar produced in Portugal as a new cow bedding material, since the use of biochar has been shown to have the potential to mitigate the impact of relevant bacterial species when added to animal manure microbiota. Our experimental setup consisted on fresh RMS samples that were collected on a commercial dairy farm and placed in naturally-ventilated containers for a total of 4 groups: 1–non-supplemented RMS; 2-RMS supplemented with 2.5% (wt/wt) of biochar; 3-RMS supplemented with 5% (wt/wt) of biochar; and 4-RMS supplemented with 10% (wt/wt) of biochar. Sampling was performed at 4 different incubation times (0, 5, 15 and 30 d) and in 2 distinct seasons: April–May (humid season) and June–July (dry season). The resulting 32 samples were subjected to DNA extraction and their microbiome profile determined through complete 16S rDNA gene sequencing using Nanopore next-generation sequencing. We observed that biochar supplementation clearly altered the microbiome of RMS, which was reflected in changes in populations' diversity and their relative abundance of relevant pathogenic bacteria. In particular, we found that long-term storage (30 d) was more beneficial than short-term storage, an effect that was more evident for samples supplemented with 2.5% or 5% biochar. In both seasons, those concentrations of biochar led to a decrease in the levels of several mastitis-causing agents (Enterobacteriaceae, streptococci, enterococci and staphylococci). In addition, we also observed a reduction in the levels of *Salmonella* spp. and Gram-positive bacilli in the biochar-supplemented samples. Unexpectedly, however, those same conditions yielded an increase in the abundance of *Brucella* spp., a group which includes important infectious agents, highlighting the need for a deeper evaluation of the impact of biochar supplementation of RMS to ensure the future safe and sustainable use of this environmental-friendly resource in animal production.
Keywords: Biochar, Dairy cows, Microbial evaluation, Recycled Manure Solids

INTRODUCTION

Cattle manure contains high concentrations of bacteria (10^9 to 10^{10} cfu/g) and fresh manure, in particular, is associated with elevated counts of Enterobacteriaceae, *Staphylococcus aureus*, and *Enterococcus faecium*, making it a potential source of pathogens and antimicrobial-resistant bacteria and genes to humans, animals and the environment (Buta-Hubeny et al., 2022). The contamination of manure with relevant bacteria is especially important in farms affected by calf diarrhea, a multifactorial disease that involves several pathogens, including *Salmonella enterica* and *Escherichia coli*, posing risks to both animal and human health (Naranjo-Lucena and Slowey, 2023).

Nevertheless, the reuse of cattle manure as bedding material for dairy cows in the form of Recycled Manure Solids (RMS) is becoming popular due to its cost efficiency and comfort when used as bedding material, offer-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

ing a sustainable and easy to handle solution that aligns with modern farming practices (Jeppsson et al., 2024). The bedding material significantly impacts dairy cattle's health, as cattle spends much time lying down, allowing the contact between the animals' ventral region, namely the udder, and bedding bacteria. High counts of environmental pathogens in this material are a risk factor for the development of several infectious diseases in dairy cows (Rowbotham and Ruegg, 2016). One of those diseases is mastitis, a mammary gland inflammation often caused by microbial infection, which poses significant health and economic challenges to the dairy industry. Common pathogens associated with bovine mastitis include *Staphylococcus aureus*, non-aureus *Staphylococcus*, *Streptococcus* species (*S. agalactiae*, *S. uberis*, and *S. dysgalactiae*), and *E. coli* (Naranjo-Lucena and Slowey, 2023). Besides, dairy cows are also susceptible to reproductive tract inflammatory diseases, such as puerperal metritis, one of the reasons for antimicrobial treatment in dairy cattle. Metritis may be caused by multiple bacteria, including *E. coli*. *Brucella abortus* is another important agent associated with reproductive tract diseases in these animals, leading to abortion and birth of weak calves, posing a threat to livestock health and longevity as well as the sustainability of dairy farms. Brucellosis is also a relevant zoonotic disease (Khurana et al., 2021; Pires et al., 2024).

To mitigate the risk of RMS usage as cow bedding, several manure pretreatments are available. These include chemical treatments, physical methods, or biological processes, but they often lead to incomplete removal of antibiotic residues and pathogens (Varma et al., 2021). As such, to address the challenges associated with cattle manure management, the use of biochar, resulting from the controlled pyrolysis of organic materials, represents a promising alternative (Meyer et al., 2011). Biochar's high surface area and porous structure enable it to adsorb a wide range of substances, including antibiotics, also having a noteworthy impact on microbial communities within manure. Namely, biochar can promote the growth and activity of beneficial microorganisms while leading to the suppression of cattle pathogens and reducing disease transmission risks to humans (Ma et al., 2024).

In light of biochar's promising potential and the cost-effectiveness of RMS use as cow bedding, our study aimed to investigate the effects of biochar on the microbial community within RMS, focusing on potential cow pathogens such as Enterobacteriaceae, *Enterococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Salmonella* sp., and *Brucella* sp. Through a pilot incubation experiment conducted across 2 seasons, RMS obtained from a dairy farm was supplemented with different biochar concentrations, and the microbiome's dynamics was analyzed post-incubation.

MATERIALS AND METHODS

Sample Collection and Processing

This research was carried out using fresh recycled manure solids (RMS) from a commercial dairy farm located in the south of Portugal, obtained through mechanical separation from fresh slurry (liquid manure) by a screw mechanism. A pilot incubation experiment was set up during 2 distinct time periods: the humid season (April–May 2022) and the dry season (June–July 2022). For that, samples comprising 5 kg of RMS were placed in naturally ventilated containers, which were assigned to 4 different groups containing: 1) non-supplemented RMS (negative control); 2) RMS supplemented with 2.5% biochar (wt/wt); 3) RMS with 5% biochar (wt/wt); and 4) RMS with 10% biochar (wt/wt). After incubation at ambient temperature for 4 distinct time periods (0, 5, 15 and 30 d of incubation), 10 g of RMS from each of the 3 replicate containers set per condition were collected, treated as a composite sample and stored at −20°C to be used in subsequent analyses. Overall, 16 composite samples were obtained per season, for a total of 32 samples for both seasons under study.

DNA extraction

The 32 samples to be analyzed were subjected to DNA extraction, which was carried out using the DNeasy PowerMax Soil Kit (QIAGEN, Venlo, the Netherlands), following the manufacturer's instructions. DNA's quality and concentration were assessed by NanoDrop One and Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, USA).

Amplification of 16S rDNA gene and Microbial Diversity Profiling through Next-Generation Sequencing

For the rDNA gene amplification Long Amp hot start Taq 2 × master mix (New England Biolabs, MA, USA) was used at 1X along with 50 ng/µL of genomic DNA from each sample. To amplify the full-length 16S rDNA bacterial gene, 0.25 µM of the primer pair 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) were used. The PCRs were conducted on a Biometra UNO II, using the following conditions: 1 cycle of 94°C for 1 min, 35 cycles of 94°C for 20 s, 55°C for 30 s, and 65°C for 2 min, and a final extension of 65°C for 5 min.

Subsequently, amplification products were visualized through gel electrophoresis and purified using the Solid phase Reversible Immobilization (SPRI) technique with

magnetic beads (DeAngelis et al., 1995; Stortchevoi et al., 2020).

Quantification steps were performed using the 1xds-DNA HS assay for Qubit. DNA was end-repaired (New England BioLabs, MA, USA), cleaned with Agencourt AMPure XP Beads (Beckman Coulter, High Wycombe, UK) and dA-tailed (New England BioLabs, MA, USA). The library was prepared from 300 ng input DNA from each sample using the Sequencing Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Oxford Nanopore Technologies, Oxford, UK) in accordance with the manufacturer's protocol. The library was quantified and prepared for PromethION sequencing, using FLO-PRO114M flow-cells, MinKNOW v22.12.4, standard 72 h run script with active channel selection enabled. After 24h yielded 3.5 million passed reads with an estimated N50 of 1500bp and the mean quality score was 14.5. In total 5.25 Gb of data were produced, with an average of 110,000 reads per sample.

Bioinformatics and Statistical analyses

The sequencing data obtained from 16S amplicons was initially preprocessed, to ensure the accuracy and reliability of the results obtained. Specifically, low quality reads were removed and only the remaining reads with lengths higher than 200 bps were retained using the Prinseq-lite tool. Moreover, reads with a Q score below 7 were also disregarded (Schmieder and Edwards, 2011). Taxonomic classification followed a Lowest Common Ancestor approach and was performed through indexing based on k-mers mapping to the lowest common ancestor of all genomes known to contain a given k-mer (Wood et al., 2019). This classification used as reference databases the NCBI RefSeq reference genomes and NCBI GenBank reference sequences of Archaea and Bacteria (up to May 2023).

Following classification, data was rarefied and subjected to: Shannon diversity index analysis (McMurdie and Holmes, 2013); α diversity group significance analysis (Bolyen et al., 2018); and sample dissimilarity analysis – Principal Coordinates Analysis (PCoA) for β diversity analysis based on the Bray-Curtis similarity index and determination of taxa abundance (with a genera prevalence cutoff of ≥0.01) (Bolyen et al., 2018). Before Shannon diversity index analysis, to account for uneven sampling depth, the data were rarefied to the minimum sampling depth of 8000 sequences. To produce the α diversity graphics (for both Shannon and Pielou's evenness indexes), the samples were randomly subsampled to create 3 technical replicates. Statistical analysis was then performed using the Kruskal-Wallis test for pairwise comparisons between each sample and the respective negative control. Significant differences were considered when the p-value (P) < 0.05. Beta diversity was evaluated by Principal Coordinates Analysis (PCoA) based on Bray-Curtis Index distance using QIIME (Bolyen et al., 2018). Only families/genera corresponding to a relative abundance higher than 0.1% were considered for analysis (Cunha et al., 2021).

RESULTS

Bacterial Populations in Biochar-supplemented Recycled Manure Solids

The results from Next-generation sequencing and subsequent taxonomical analyses revealed a distribution of each level that corresponded to a total of 23 phyla, 41 classes, 85 orders, 195 families, 467 genera and 862 species for the samples collected in the humid season and a total of 48 phyla, 82 classes, 166 orders, 358 families, 1001 genera and 2117 species for the samples collected in the dry season (Supplemental Tables S1 and S2; https://doi.org/10.7910/DVN/JFOSKC). These results evidenced a clear difference in taxonomic richness between the samples collected in both seasons, with the samples of the dry season having twice the number of all taxonomic levels on average when compared with the samples of the humid season. This provided us with a first indication that the samples of the dry season had a more complex bacterial population than the one found in the humid season samples.

The β diversity analysis shown in Figure 1 allows us to observe that for both seasons the initial samples collected were similar but started to diverge with time. However, that evolution in microbial populations seemed to progress faster in the dry season than in the humid season, as evidenced by the fact that the samples collected on d 15 and 30 in the dry season were much more similar among themselves than those from the humid season. Seeing that population dynamics tended to stabilize only for the longer incubation period, moving forward we will mostly focus on the data obtained for those time points (d 30).

Next, we assessed the α diversity of the samples under study, for both seasons, using Pielou (Figure 2a and c; Supplemental Tables S3 and S4; https://doi.org/10.7910/DVN/JFOSKC) and Shannon indexes (Figure 2b and d; Supplemental Tables S3 and S4), which assess the evenness of the distribution and the overall diversity of microbial types within a population, respectively. After 30 d of incubation during the humid season, the average Pielou index of the negative control was 0.86, while that of biochar-supplemented samples was 0.82, 0.81 and 0.82 for the samples with 2.5%, 5% and 10% of biochar, respectively. The corresponding values of those samples for the Shannon index were 7.95, 7.24, 7.37 and 7.52. Therefore, biochar addition led to a statistically sig-

nificant decrease (p-value = 0.0495) in both the evenness and diversity of the microbial populations present in the biochar-supplemented samples. We observed the exact same effect in the biochar-supplemented samples in the dry season, except for the 10% biochar-supplemented sample that presented a significantly lower evenness (p-value = 0.0495) than the negative control but exhibited no significant differences in the Shannon index (p-value = 0.512). In general, biochar-supplementation had a significant impact in the populations' α diversity, leading to lower species richness in those samples. Remarkably, the results obtained also pointed to an overall higher diversity in the dry season, since the Shannon index was found to be, on average for the 4 samples analyzed at 30 d of incubation, about 8% higher in the dry season than in the humid season (values of 8.09 and 7.52, respectively), even though the Pielou index exhibited a slight decrease (of approximately 5%). This is in accordance with the higher number of taxonomic levels mentioned above (Supplemental Tables S1 and S2), which had already suggested the existence of more diverse bacterial populations in the dry season.

Main Causative Agents of Bovine Mastitis Present in RMS

Considering our goal of evaluating whether biochar supplementation could promote changes in the pathogenic RMS microbiome, we initially set out to assess the relative abundance of the most common causative agents of bovine mastitis in the RMS samples. We detected the presence of the most relevant genera known to cause mastitis in cows in all the samples, including members of the *Escherichia, Streptococcus*, *Enterococcus,* and *Staphylococcus* genera. Remarkably, the relative abundance of all these genera was found to decrease at 30 d of incubation, in both seasons, in the biochar-supplemented samples, particularly those containing 2.5% and 5% of biochar. In the humid season, where the differences were more pronounced, the percentage of reduction in the samples containing 2.5% and 5% of biochar, when compared with the negative control, was as follows (respectively): 29.71% and 41.48% in members of the Enterobacteriaceae family, with a 53.55% and 68.96% decrease in *Escherichia* spp.; 7.1% and 53.44% in species belonging to the *Enterococcus* and *Streptococcus* genera; and 83.61% and 83.57% in staphylococci (Figure 3; Supplemental Tables S5 and S6; https://doi.org/10.7910/DVN/JFOSKC). In the dry season, the decrease observed in the relative abundance of these genera in the equivalent samples (with 2.5% and 5% biochar) was: 39.82% and 25.51% in Enterobacteriaceae, and 42.88% and 22.54% in *Escherichia* spp.; 18.76% and 33.21% in enterococci and streptococci; and 65.28% and 66.37% in staphylococci (Figure 4; Supplemental Tables S5 and S6).

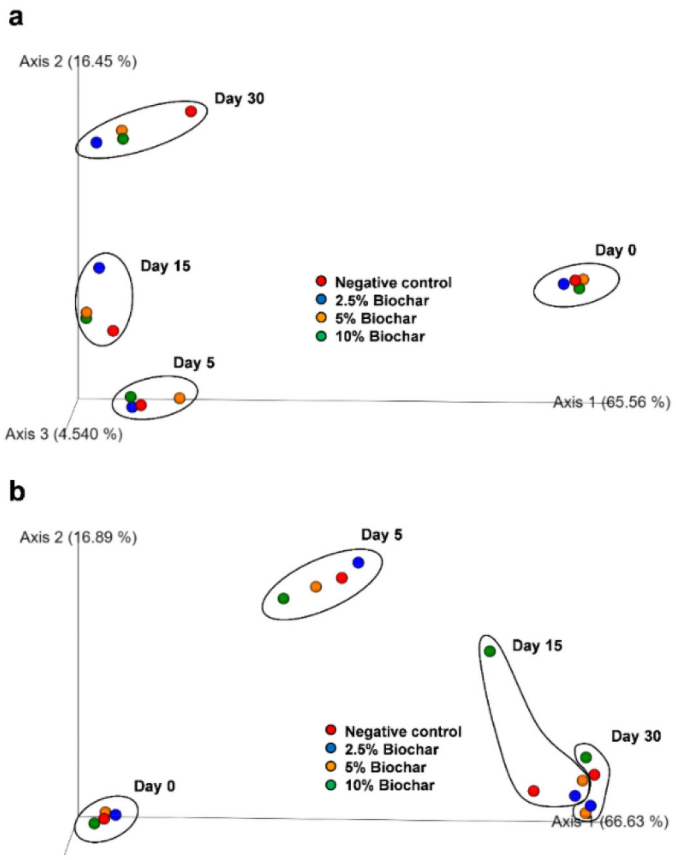

Figure 1. Principal Component Analysis (PCA) of bacterial communities in the humid (a) and dry (b) seasons at the different times of incubation and under all the conditions tested in this study.

Other Relevant Agents from a One Health Perspective Present in RMS

Since the use of RMS as cow bedding has implications that go beyond the scope of bovine health, presenting a potential environmental and human threat, we also focused the analysis on bacterial genera that are relevant from a One Health perspective. In that context, one discriminative difference observed was the marked reduction observed in the relative abundance of spore-forming Gram-positive bacilli in biochar-supplemented RMS samples (Figures 5a and 6a; Supplemental Tables S5 and S6). Once more, this divergence was more evident in the humid season, with the combined relative abundance of members of the *Bacillus* and *Clostridium* genera exhibiting a decrease of 99.58%, 55.42% and 60.32% in the 2.5%, 5% and 10% biochar-supplemented samples, respectively, when compared with the negative control. While more modest, an overall decrease of 26.52% was

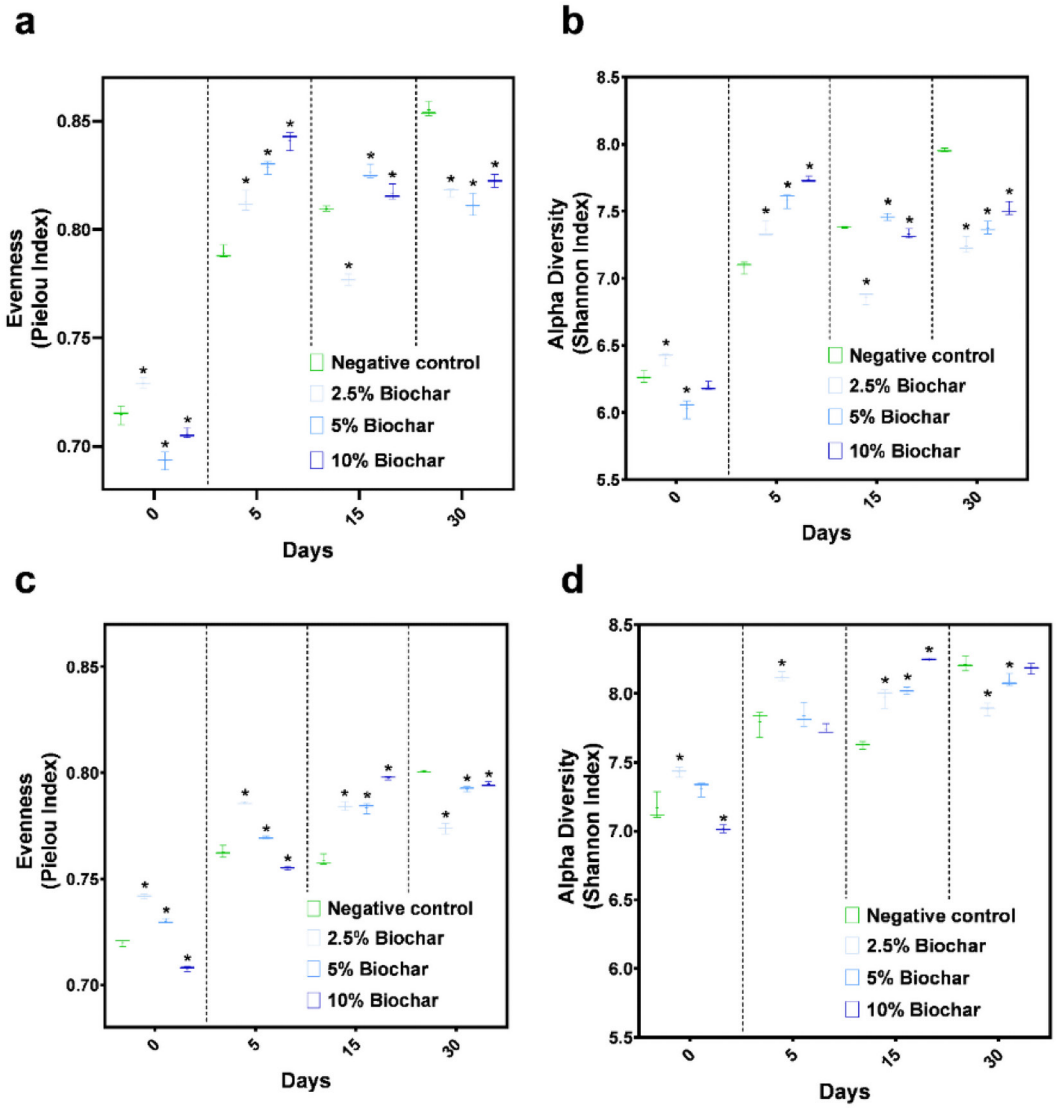

Figure 2. Alpha diversity boxplots of bacterial communities in the humid (a, b) and dry (c, d) seasons at the different times of incubation and under all the conditions tested in this study. Bacterial evenness estimated by the Pielou index (a, c) and bacterial diversity estimated by the Shannon index (b, d) in the humid and dry seasons, respectively. Statistical analysis was performed using the Kruskal–Wallis test and a significant p-value is represented by an asterisk (*p-value <0.05).

also observed for the 3 biochar-supplemented samples from the dry season. Additionally, one other pathogen, relevant in terms of gastrointestinal health in both humans and cattle, was found to have decreased abundance in the samples where biochar had been added. *Salmonella* spp. were found to be, on average, 42.27% and 29.98% less abundant in the biochar-supplemented samples than in the control sample, in the humid and dry seasons, respectively (Figures 5b and 6b; Supplemental Tables S5 and S6). This was particularly relevant since *Salmonella* was the most abundant genus belonging to the Enterobacteriaceae family detected in this study in samples from both seasons.

Despite the promising results mentioned so far, not all changes observed in biochar-supplemented samples were desirable. Unexpectedly, we observed an increase in the relative abundance of 2 known bovine pathogens, which are also relevant pathogens in other species, including humans. Specifically, in the humid season, members of the *Pseudomonas* and *Brucella* genera were found to have a very noteworthy upsurge in biochar-supplemented samples, particularly in the sample supplemented with 2.5% biochar (Figure 5c and d; Supplemental Tables S3 and S4), where they rose 283.99% and 110.25%, respectively, in relation to the negative control. In the dry season, the relative abundance of *Pseudomonas* spp. showed

the opposite trend, decreasing 31.15% in the 2.5% biochar-supplemented sample, but the levels of *Brucella* spp. remained much higher (by 67.06%) than those of the negative control (Figure 6c and d; Supplemental Tables S5 and S6).

DISCUSSION

This study was carried out to elucidate the potential of biochar supplementation in increasing the safety of Recycled Manure Solids (RMS) used as bedding material for dairy cows. The sustainable aspect of this work focused not only on reusing manure solids as a cost-effective and environmentally-friendly bedding strategy, but also on the use of a pine biochar produced locally in Portugal. However, before being used as bedding material, RMS have to comply with microbiological requirements. In that context, we collected RMS samples, which were then treated (or not, as a control) with different concentrations of biochar and incubated at ambient temperature for up to 30 d, before we characterized their microbiome using next-generation sequencing. The data obtained revealed

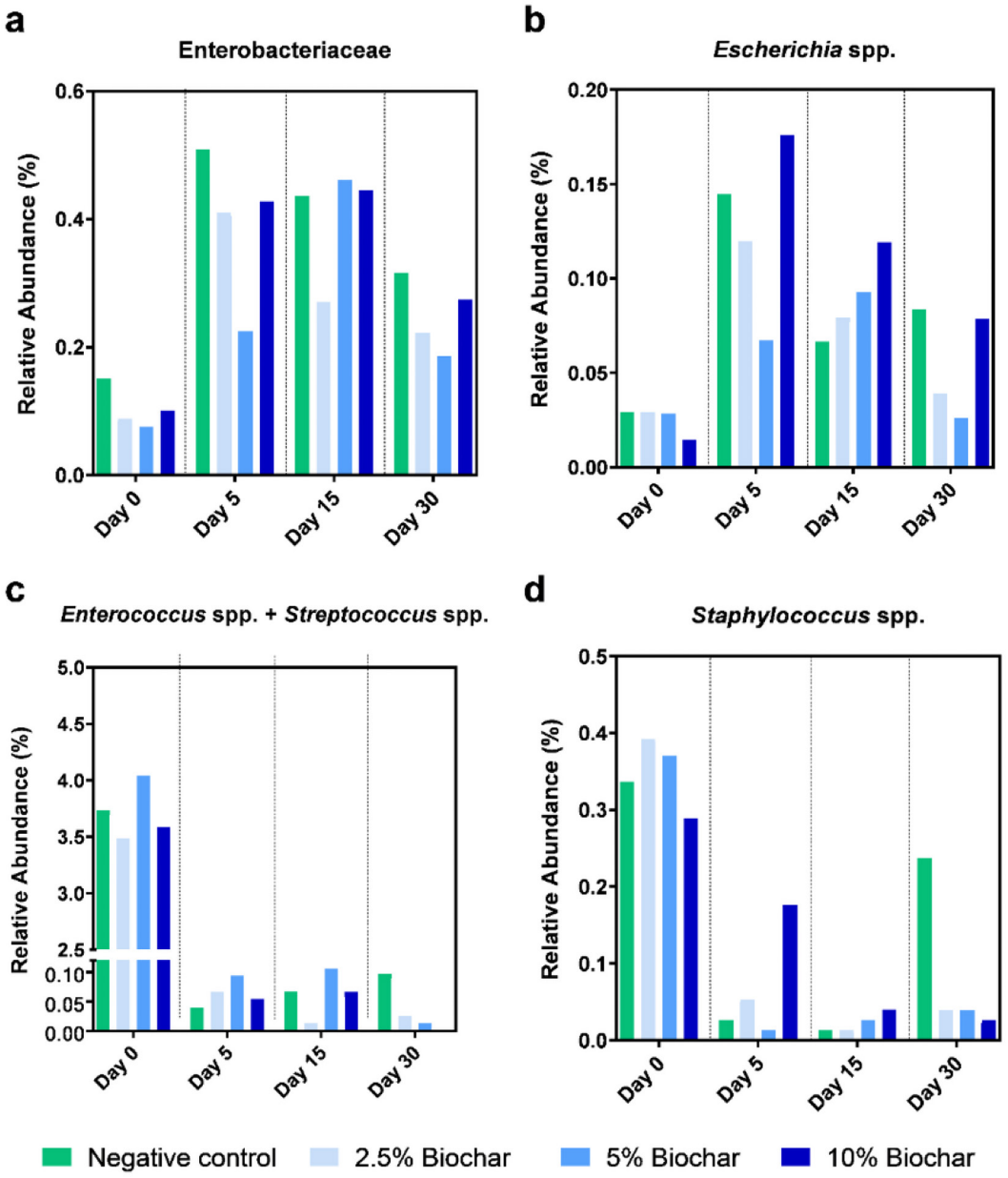

Figure 3. Relative abundance of selected families (a) and genera (b, c, d) found in the samples collected in the humid season, which are relevant to the development of bovine mastitis.

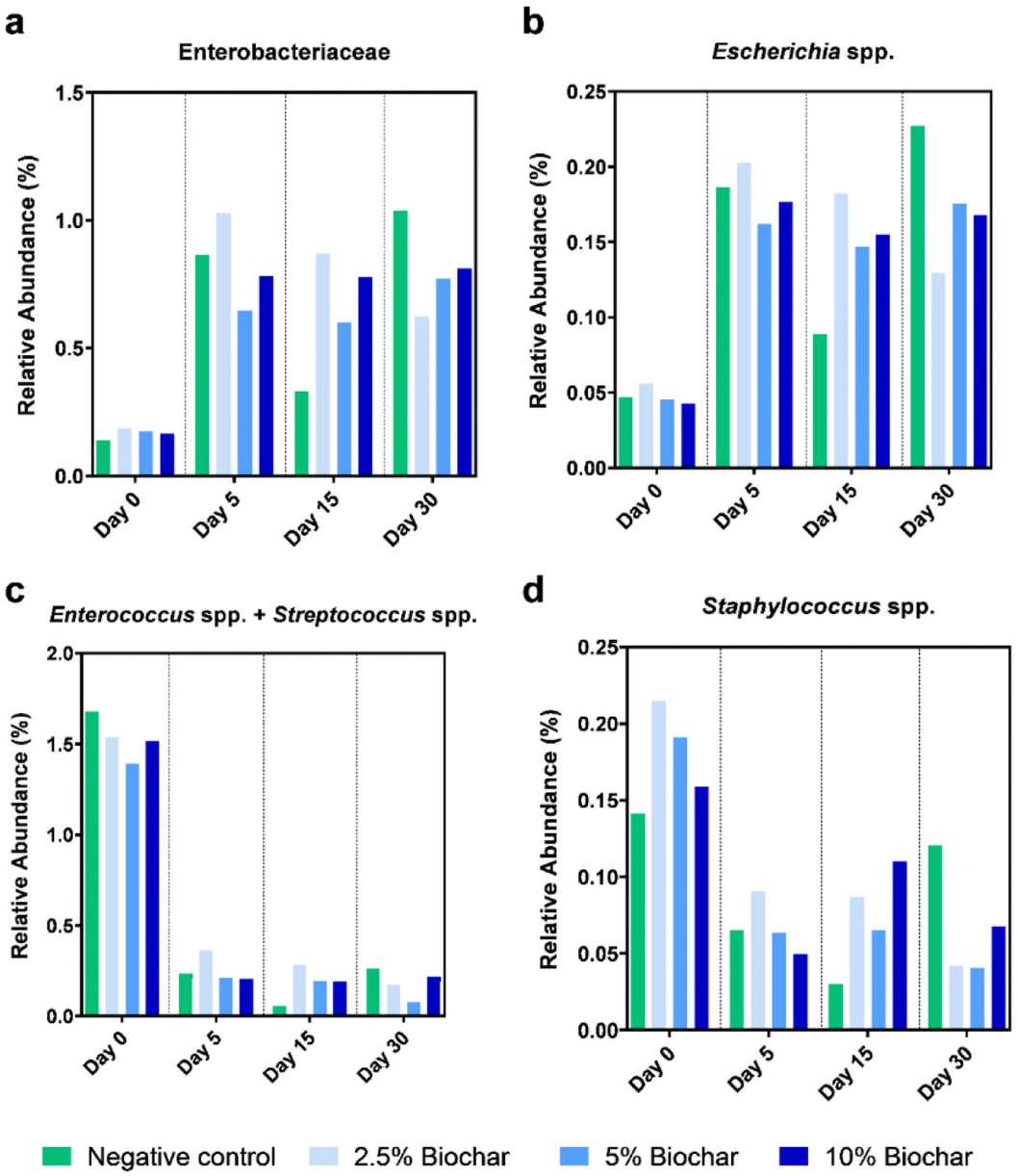

Figure 4. Relative abundance of selected families (a) and genera (b, c, d) found in the samples collected in the dry season, which are relevant to the development of bovine mastitis.

a complex series of changes in the bacterial dynamics of biochar-supplemented samples that shed some light on the potential benefits, but also on some limitations, of the use of this new bedding material.

The first objective was to evaluate the presence of several important mastitis-causing pathogens in RMS samples and whether biochar could play a role in reducing the levels of those pathogens. This is particularly relevant in the case of RMS-based bedding materials, since it has previously been shown that even though the bacterial load present in composted RMS decreases when compared with fresh RMS (namely that of coliforms and *Streptococcus* spp.), several mastitis pathogens are not eradicated, maintaining bacterial counts that can be problematic (Cole and Hogan, 2016). In this study, we did observe a decrease in the levels of *Streptococcus/Enterococcus* and *Staphylococcus* throughout time, but not in the Enterobacteriaceae level.

Moreover, fresh manure solids have been demonstrated to have higher total bacterial numbers before use than other fresh bedding materials, such as sand, recycled sand, paper fiber and straw (Bonhotal et al., 2010; Alanis

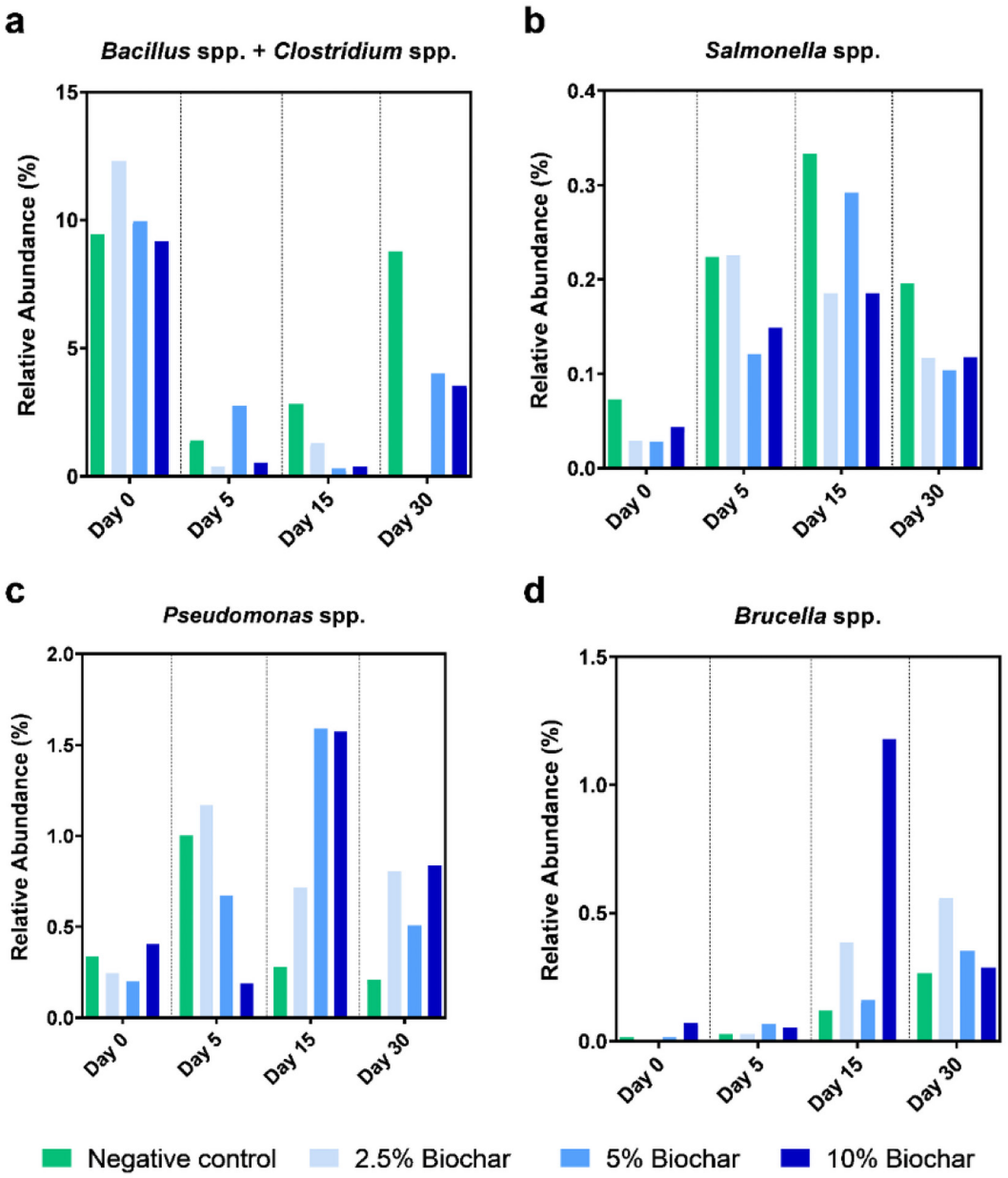

Figure 5. Relative abundance of selected genera found in the samples collected in the humid season, which are relevant in a One Health perspective.

et al., 2021). While average bacterial counts generally increase for all bedding types after use, used manure solids were found to have higher levels of streptococci than used and recycled sand; of coliforms than used paper fiber, sand and recycled sand; and of non-coliforms than all those types of beddings after use (Alanis et al., 2021). According to our data, we were unable to completely eradicate these pathogens in RMS using biochar supplementation, since we detected the presence of several members of the *Escherichia*, *Streptococcus*, *Enterococcus,* and *Staphylococcus* genera, even in samples that were incubated with biochar for as long as 30 d (the exception being the 10% biochar-supplemented sample in the humid season in which no enterococci or streptococci were detected). In fact, it would be very difficult to eliminate these pathogens using this type of additive, and the use of more aggressive methods could compromise the subsequent use of RMS as cow bedding. As such, it is particularly encouraging that, in this work, we observed a very marked decrease in the level of all of those previously mentioned pathogens. In the humid season, the most beneficial effect was observed for the samples sup-

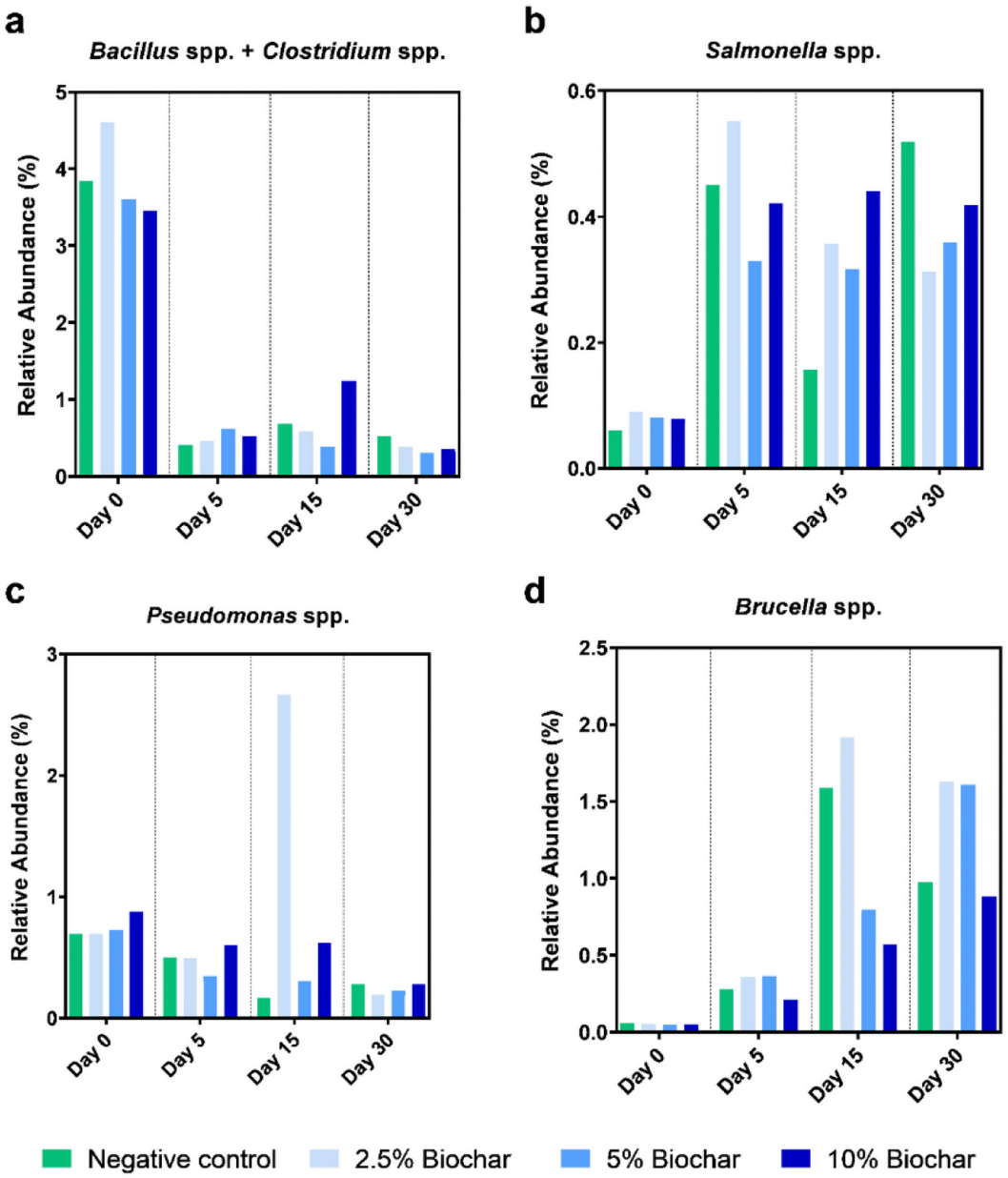

Figure 6. Relative abundance of selected genera found in the samples collected in the dry season, which are relevant in a One Health perspective.

plemented with 5% of biochar, which led to an average reduction of 59.50% of Enterobacteriaceae, streptococci, enterococci and staphylococci as a whole. In the dry season, the supplementation with 2.5% or 5% of biochar had similar effects, leading to an overall decrease of these 4 groups by 41.29% or 41.70%, respectively. The main difference between the use of these concentrations was that 2.5% of biochar led to a higher reduction in Enterobacteriaceae levels, while 5% of biochar was more effective in reducing the levels of streptococci/enterococci. This is in agreement with a report from other authors that has shown that pine biochar application to poultry litter led to a significant reduction in *E. coli* and total aerobic bacteria counts (Mohammadi-Aragh et al., 2022). In most of the scenarios analyzed, the use of a higher concentration of biochar (10%) didn't seem to yield better results than the use of lower concentrations (2.5% and 5%), demonstrating that the best overall performance could be obtained using the most cost-effective hypotheses.

Interestingly, previous research has reported that some strategies, such as replacing RMS daily from the back one-third of cow stalls, reduced cow's exposure to co-

liforms, but was ineffective against *Streptococcus* spp. (Sorter et al., 2014). Since composted RMS has been shown to have reduced bacterial counts before use, but that effect is soon lost as bacterial counts rise drastically after use, the emphasis should probably be put in the management of bedding once in use (Bonhotal et al., 2010; Sorter et al., 2014). In that sense, biochar might represent an advantage when added to RMS before use, but mainly during its usage in stalls, as it can decrease the levels of relevant pathogens on site, which seems to be the main factor needed to protect the udder from exposure to mastitis-causing pathogens.

It has been shown by many authors that there is a clear positive correlation between the dry mater bedding content and the growth of *Streptococcus* spp., coliforms, and non-coliforms and incidence of environmental clinical mastitis (Fávero et al., 2015; Alanis et al., 2021; Freu et al., 2023). Seeing that bed moisture plays such an important role in the development of mastitis, we performed our pilot experiment in 2 different seasons, to account for the effect of seasonality on the effectiveness of biochar's supplementation of RMS. Remarkably, we observed the largest decrease in causative agents of mastitis in the humid season, which is fairly promising since this is the season in which keeping the bedding drier represents a bigger challenge, as the relative environmental humidity was much higher (71.5%) than in the dry season (56.3%). We also observed that dry season samples presented a higher microbial diversity than the humid season samples, which could be related with the fact that it was in the dry season that we observed the highest overall variation in both temperature and humidity (of 12.6°C and 56.0% as opposed to 9.5°C and 43.0% variation in the humid season). It is possible that biochar could be overall more effective in the humid season, decreasing or increasing more drastically the levels of other bacteria in the community, thus leading to less diverse communities with less evenly distributed genera. Consistent with a previous report that assessed the effect of biochar addition on cow manure composting, we also observed that biochar supplementation led to a decrease in the diversity of the microbial populations under study (Ma et al., 2024).

Two additional positive effects of biochar addition to RMS observed in our work were the decreased levels of *Salmonella* spp. and Gram-positive bacilli found in supplemented samples, evidencing an interesting additional effect of biochar in reducing the level of known foodborne pathogens or spore-forming bacteria that can constitute a hazard even in pasteurized milk. While previous studies have determined that the use of RMS bedding did not lead to an increased presence of *Salmonella* spp., *Bacillus cereus* or bacterial spores in milk, those pathogenic agents were still found to be present in a small amount of samples (Bradley et al., 2018; Gagnon et al., 2020). Considering the risk that these pathogens of zoonotic interest may pose, in particular when one considers the ability of bacterial spores to resist high temperatures, including the ones used in milk pasteurization, it is particularly encouraging that biochar exhibited such an effective result in the reduction of these bacterial genera. Use of RMS bedding was also reported to increase the risk of detecting thermoresistant streptococci and enterococci in milk, which could have an important impact in the food industry, for instance by affecting the organoleptic properties of cheese (Gagnon et al., 2020). In this context, biochar can also help to prevent or minimize that risk, since it displayed a good efficacy in the reduction of the levels of streptococci and enterococci in RMS, according to the results obtained in our study.

Despite all the promising insights into the potentially beneficial role of biochar addition to RMS discussed so far, our study also revealed some undesirable effects. The most significant was the very marked increase observed in the levels of *Brucella* spp. in biochar-supplemented samples. Albeit surprising, this stimulating effect of biochar-like substances on *Brucella* growth had already been described as early as in 1951, when *Brucella suis* was cultivated in a system based on charcoal and cellophane, and activated charcoal continues to be used in the development of growth medium selective for *Brucella* (Gorelick et al., 1951; Mena-Bueno et al., 2022). At the time of collection of the RMS samples used in this study, we had no knowledge that *Brucella* spp. were circulating in the dairy farm in question. However, a brucellosis control program performed later in the year, in November 2022, led to a few animals testing positive for brucellosis. Considering the health and economic constraints this disease represents for a dairy farm, routinely screening cow bedding for the presence of specific pathogens might present an opportunity for early detection and subsequent prevention of higher losses.

The second troublesome effect we observed after biochar addition was an increase in the relative abundance of members of the *Pseudomonas* genus in the humid season, which includes species that are relevant bovine and human pathogens, such as *P. aeruginosa*. However, in the dry season, the opposite effect was observed. This is somewhat in agreement with what is found in the literature, where conflicting effects have been reported in this matter. While a recent study has shown that biochar addition to cow manure promoted its maturity through the reduction of the abundance of *Pseudomonas* spp., another study suggested that biochar may promote quorum sensing and biofilm formation in *P. aeruginosa*, which would make them thrive and be harder to eliminate from the environment (Yan et al., 2023; Ma et al., 2024). Further studies are thus needed to understand these dynamics and the factors that play a role in this process,

even though the seasonality of this event observed in our study might indicate that humidity or temperature might be crucial. In addition, overall, these results highlight that while RMS supplementation with biochar can be an effective way to reduce the level of some relevant pathogenic species, its use should be further investigated, in particular to account for off target, undesirable effects that can compromise its efficacy and safety.

One important limitation of the present study is that the metagenomic analysis performed detects environmental DNA and not the pathogens directly. As such, in the future, it would be important to further investigate the potential of biochar in modulating the level of relevant pathogens, both in supplemented and non-supplemented manure samples, and in milk samples of animals with mastitis, to better understand the potential benefits of the findings described herein, namely regarding a useful effect of biochar on decreasing exposure to mastitis-causing pathogens to the dairy farm from which the RMS samples were sourced.

NOTES

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All animals were cared for according to the rules given by the current EU (Directive 2010/63/EC) and national (DL 113/2013) legislation and by the competent authority (Direção Geral de Alimentação e Veterinária, DGAV, www.dgv.min-agricultura.pt/portal/page/portal/DGV). Only noninvasive samples were collected during routine procedures with consent of owners, and no ethics committee approval was needed. Trained veterinarians obtained all the samples, following standard routine procedures. No animal experiment has been performed in the scope of this research. Verbal informed consent was obtained from all the owners, all the necessary information about the study was provided to all the participants before obtaining their consent.

ABBREVIATIONS: RMS = Recycled Manure Solids

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