

# Effect of sodium bisulfate amendments on bacterial populations in broiler litter

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**ABSTRACT** The accumulation of ammonia in poultry houses is of concern to bird and human health. Acidification of the litter by application of acidifying amendments such as sodium bisulfate (**SBS**) retains ammonia generated by microbial degradation of uric acid as harmless ammonium in the litter. Although some studies on the effects of litter amendments on specific bacteria and groups of bacteria have been carried out previously, wide gaps in knowledge remain. In the present study, 2 types of samples were prepared and either left unamended or amended with 2.5 or 10% SBS. One set of samples consisted of a 1:1 mixture of built-up litter and fresh poultry manure (**L/M**); the other of fresh wood shavings and fresh poultry manure (**S/M**). The samples were kept in the laboratory at room temperature for 35 d. The pH of unamended mixtures increased to 7.3 and 6.9 for L/M and S/M, respectively. A pH of 6.7 and 3.9 on day 35 was observed for L/M and SM with 2.5% SBS, respectively. The corresponding values for LM and SM amended with 10% SBS were 3.5 and 2.5, respectively. Plating data

indicated that coliforms became less numerous in the unamended samples than the SBS-amended samples. This difference was also seen in data obtained by high-throughput sequencing of 16S rDNA. The sequencing data also indicated that sequences from the genus *Oceanisphaera* accounted for as much as 80% of the sequences from L/M and about 40% of those from S/M samples early on. Sequences from members of the order Clostridiales were enriched in L/M and S/M amended with 10% SBS as were sequences from the genus *Turcibacter*. *Weisella* species sequences were more prevalent in SBS-amended samples than in unamended ones. Sequences from the genus *Corynebacterium*, *Brachybacterium*, and *Arthrobacter* were more common in L/M samples than in S/M samples regardless of the SBS content. The data indicate that litter amendments affect some bacteria populations and not others. Further studies are required to determine if the observed population changes such as increased survival of coliforms warrant actions to improve the microbial quality of litter to be reused.

**Key words:** broiler, litter, bacteria, amendment, sodium bisulfate

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## INTRODUCTION

Depending on its age and poultry husbandry practices, poultry litter is made up of more or less inert bedding materials such as wood shavings and various levels of organic and inorganic matter produced by the birds housed on the litter. Microorganisms present in the litter utilize organic compounds provided the moisture content of the litter is sufficiently high. Organic nitrogen compounds, primarily uric acid excreted by the birds, are degraded, yielding urea and finally ammonia. Some of the ammonia volatilizes and can negatively

impact the health and well-being of the birds as well as that of human workers. The volatilizing of ammonia generated by the microorganisms can be reduced by amending the litter with acidifying compounds such as aluminum sulfate (alum) and sodium bisulfate (**SBS**), thus retaining more of the ammonia as its ammonium salt (Moore et al., 2000; Miles et al., 2006).

Poultry litter without such amendments can have pH values of 8.5 (Moore et al., 2000; Miles et al., 2006) and could be expected to harbor microbial populations that are adapted to this environment. Similarly, amended litter with a lower pH will likely be populated by microorganisms that thrive under those conditions. Some studies have looked at differences in specific bacterial populations inhabiting litter with and without amendments. For example, Line (2002) observed that raising chickens on litter treated with aluminum sulfate or SBS reduced *Campylobacter* colonization but did not impact *Salmonella* levels. Sodium bisulfate was able to reduce *Salmonella*

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**Table 1.** Moisture and pH of fresh manure, wood shavings, and litter used.

Parameter	Fresh manure	Shaving	Litter	SEM	Significance (P = )
Moisture, %	74.3 <sup>a</sup>	7.8 <sup>b</sup>	15.2 <sup>c</sup>	0.105	<0.001
pH	6.0 <sup>a</sup>	7.2 <sup>b</sup>	7.8 <sup>c</sup>	0.70	<0.001

<sup>a-c</sup>Means within rows not sharing common suffixes are significantly different at the 5% level of probability.

Typhimurium levels in litter when assayed 1 d after application of the amendment provided the amendment levels were high enough to reduce the litter pH to 5.5 and below (Payne et al., 2002). In contrast, Williams et al. (2012) observed that with a decreasing pH because of SBS addition, *Salmonella* applied as a cocktail of 5 serovars was able to survive better in amended than unamended litter. Rothrock et al. (2008), using a bird-free system, observed lower *Campylobacter* counts in alum-treated litter than in untreated litter. *Escherichia coli* counts were also lower. These authors also studied the effect of the amendment on bacterial and fungal populations using denaturing gradient gel electrophoresis, a method not based on culture. Results for these assays indicated a reduction in *Clostridium/Eubacterium* and low %GC gram-positive bacteria in amended litter. *Actinomyces* sp. were not affected. The results of the denaturing gradient gel electrophoresis analyses also indicated differences in the fungal communities of treated and untreated litter. Choi et al. (2008) studied the effect of alum, aluminum chloride, and ferrous sulfate on total aerobic and bacteria able to grow on McConkey agar (gram-negative bacteria) and found lower total and gram-negative counts in litter amended with the acidifiers. Using quantitative real-time PCR, Cook et al. (2011) were able to show that acidifier amendments led to a lower number of total bacteria but a higher number of fungi. Urease-producing bacteria counts were lower in treated litter as were uricase producers in most cases. Thus, it is likely that the lower ammonia emissions observed initially after acidifier applications are not only because of ammonia being retained as ammonium ion in the litter but because of a reduction in ammonium-producing bacteria. A follow-up study with aluminum sulfate confirmed that litter

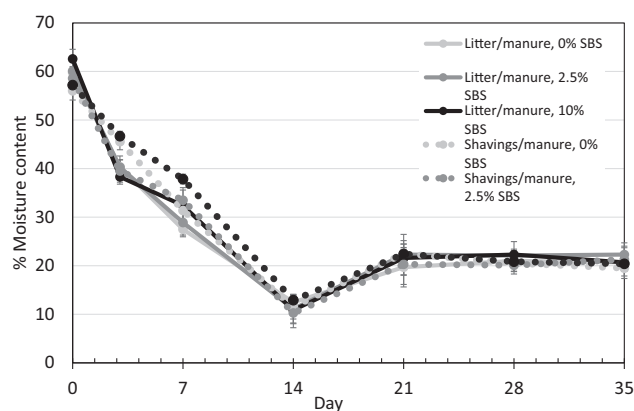
acidification reduced total bacterial populations by 50% and urease-producing bacteria by 90% within 4 wk after application to litter (Cook et al., 2008). In another study, SBS caused little change in the total bacterial population over a 56-day period, an approximately 1-log decrease in urease-producing bacteria and a small increase in uricase-producing bacteria; however, fungal counts increased about 10,000-fold (Cook et al., 2011).

The current data on the effects of acidifying litter amendments still have significant gaps, particularly with respect to the fate of bacteria of concern to poultry health and food safety. In the present study, wood shavings-based litter from a poultry house that had been populated repeatedly by chicken flocks and fresh wood shavings were mixed with fresh poultry manure at a 1:1 ratio and either left untreated or treated with 2.5 or 10% SBS. Total aerobic and coliform counts were determined weekly, and samples were taken for the extraction of DNA to be used for high-throughput sequencing. Urease activity in the different types of litter was also measured.

## MATERIALS AND METHODS

### Litter Preparation and Incubation

Two types of litter samples were prepared for the trials. The first type consisted of a 1:1 (w/w) mixture of built-up litter from a commercial broiler house and fresh broiler manure. This mixture was considered to represent the condition of birds raised on built-up litter. The commercial broiler house raised 7-week antibiotic-free broilers with SBS as standard method for ammonia control in brooding chambers. The built-up litter was collected from a non-brooding chamber before chick placement, where no SBS was used, and 3 flocks of broilers were raised on the built-up litter. The fresh broiler manure was collected in the same house with 3-week old broilers (Ross 708) on a plastic film over a 24-hour period. The second type consisted of 1 part fresh pine shavings and 1 part poultry manure and represented litter conditions with fresh bedding. The moisture contents of freshly prepared mixtures were adjusted to around 60% that was the typical level of broiler droppings on floor. The mixed materials were divided into 3 portions, 1 kg each. One portion did not receive a litter treatment agent, 1 portion received SBS (2.5% w/w), and the third portion received 10% SBS. The 2 concentrations were chosen to mimic a 25 and 100 lb/m<sup>2</sup> application rate under commercial conditions. Each treatment sample was placed into stainless steel tubs with a depth of 2.5 cm and kept at room temperature (20°C) in the laboratory with 40 to 50% relative



**Figure 1.** Moisture content of litter/manure and shavings/manure samples with and without the addition of sodium bisulfate (SBS). Data points are averages of 3 measurements, and error bars indicate SD.

**Table 2.** Moisture (%) of samples with different SBS treatment at different time points.

Treatment	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Shaving-0% SBS	56.0	45.6	31.4	11.5	22.5	20.5	19.5	20.2
Shaving-2.5% SBS	58.6	39.5	33.5	10.3	20.3	20.1	21.3	21.5
Shaving-10% SBS	57.2	46.7	37.8	12.9	22.4	20.8	20.4	21.7
Litter-0% SBS	59.8	40.4	27.5	12.5	19.7	21.0	20.7	22.0
Litter-2.5% SBS	60.1	40.3	28.9	11.1	22.3	22.1	22.3	21.5
Litter-10% SBS	62.6	38.3	32.4	10.9	21.6	22.3	20.7	22.4
SEM	3.04	3.39	3.13	3.86	4.57	3.28	3.22	3.65
Significance (P = )	0.20	0.05	0.05	0.96	0.95	0.95	0.93	0.98

Abbreviation: SBS, sodium bisulfate.

humidity for the duration of the 5-week trial. The moisture content of each sample was determined by the oven drying method on day 0, 3, 7, and weekly thereafter. Because the moisture levels declined to around 10% by day 14, deionized water was sprayed daily on the litter to increase the moisture level to approximately 20% to 25%.

### Total Aerobic and Coliform Counts

On day 0, 3, 7, 14, 21, 28, and 35, samples of approximately 20 g were taken from each pan, placed into stomacher bags, and the exact weight of each sample was determined. Nine mL of phosphate-buffered saline (PBS) per g of litter sample was added to each stomacher bag. Starting with the samples from day 7, the pH of samples with 10% SBS were adjusted immediately upon addition of PBS to between 6.5 and 7.5 with 1 M KOH as it was realized that otherwise the bacteria were exposed to a likely lethal pH. The samples in PBS or pH-adjusted PBS were subjected to stomaching for 2 min, and the homogenate was filtered through 1 layer of sterile cheese cloth. An aliquot of the filtrate was used for 10-fold serial dilutions in PBS. One mL of the filtrate and dilutions was pipetted onto total aerobic count and coliform count petrifilm (3M, Maplewood, MN), and the films were incubated at 37°C for 24 h, and colonies were counted.

### Urease Activity Determination

The QuantiChrome™ Urease Assay Kit (Bioassay Systems, Hayward, CA) was employed for the determination of urease activity in litter samples. An aliquot was removed from the litter filtrate and diluted 100-fold. One hundred eighty microliter of the suspension was

pipetted into a microcentrifuge tube, 4 µL of toluene was added, and the tube was vortexed for 15 s. The ratio of toluene to sample volume was based on the ratio used by Klose and Tabatabai (1999). Twenty microliter of urea solution from the kit was added, and the mixture was incubated for 1 h at 37°C. The tubes were centrifuged at 10,000 × g for 2 min, and 100 µL of the supernatant was used to determine urease activity according to the protocol provided by the supplier of the kit. The activity was expressed as µmoles of ammonia produced per h per g of dry litter.

### Ammonium and Nitrate Determination

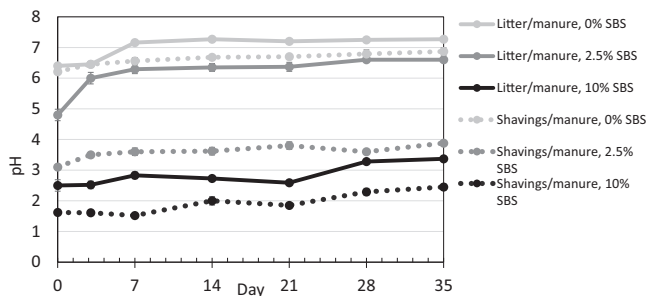
Ammonium and nitrate levels were determined by the cadmium reduction method at the Soil Testing laboratory of the University of Delaware.

### DNA Extraction and Sequence Analysis

The filtered litter samples (approximately 150 mL) were centrifuged at 10,000 × g for 30 min, the supernatant was decanted, and 250 mg of pellet material were transferred to a microcentrifuge tube and frozen at -80°C. The DNA extraction was accomplished using the DNeasy PowerSoil kit following instructions from the supplier (Qiagen, Germantown, MD). The DNA obtained was quantified using a nanodrop instrument and sent to RTL Genomics (Lubbock, TX) for amplification of 16S rRNA sequences and sequence determination using Illumina 454 technology. The sequences obtained were checked for quality, paired, and assigned to taxonomic groups by RTL. A table (supplemental data) generated from the sequence comparison data containing the numbers of sequences associated with the different phylogenetic groups was used for analyses of the changes of sequence frequencies throughout the 5-week trial.

### Statistical Analysis

Three random subsamples were taken from each treatment for pH and moisture level measurement, whereas only single composite samples from each treatment at each time point were used ammonia and nitrite, bacteria count, enzyme activity, and microbial population analysis. Statistical analysis was performed to compare pH and moisture levels among 6 treatments at each time point with 3 replicates. A multiple comparison was



**Figure 2.** pH measurements of litter/manure and shavings/manure samples with and without sodium bisulfate (SBS) supplementation over the 35-Day experimental period. Data points represent averages of 3 measurements, and error bars indicate SD.

**Table 3.** pH of samples with different SBS treatment at different time points.

Treatment	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Shaving-0% SBS	6.21 <sup>a</sup>	6.45 <sup>a</sup>	6.56 <sup>b</sup>	6.68 <sup>b</sup>	6.70 <sup>b</sup>	6.79 <sup>b</sup>	6.87 <sup>b</sup>	6.87 <sup>b</sup>
Shaving-2.5% SBS	4.30 <sup>c</sup>	3.50 <sup>c</sup>	4.32 <sup>c</sup>	3.22 <sup>c</sup>	3.80 <sup>c</sup>	3.60 <sup>c</sup>	3.88 <sup>c</sup>	3.93 <sup>c</sup>
Shaving-10% SBS	2.30 <sup>e</sup>	1.61 <sup>e</sup>	1.52 <sup>e</sup>	2.00 <sup>e</sup>	1.85 <sup>e</sup>	2.29 <sup>d</sup>	2.45 <sup>e</sup>	2.52 <sup>e</sup>
Litter-0% SBS	6.40 <sup>a</sup>	6.45 <sup>a</sup>	7.16 <sup>a</sup>	7.27 <sup>a</sup>	7.20 <sup>a</sup>	7.25 <sup>d</sup>	7.27 <sup>a</sup>	7.28 <sup>a</sup>
Litter-2.5% SBS	4.80 <sup>b</sup>	6.00 <sup>b</sup>	6.29 <sup>b</sup>	6.35 <sup>b</sup>	6.37 <sup>b</sup>	6.60 <sup>b</sup>	6.60 <sup>b</sup>	6.65 <sup>b</sup>
Litter-10% SBS	2.80 <sup>d</sup>	2.52 <sup>d</sup>	2.83 <sup>d</sup>	2.73 <sup>d</sup>	2.59 <sup>d</sup>	3.28 <sup>c</sup>	3.37 <sup>d</sup>	3.50 <sup>d</sup>
SEM	0.17	0.15	0.14	0.15	0.13	0.13	0.11	0.09
Significance (P = )	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

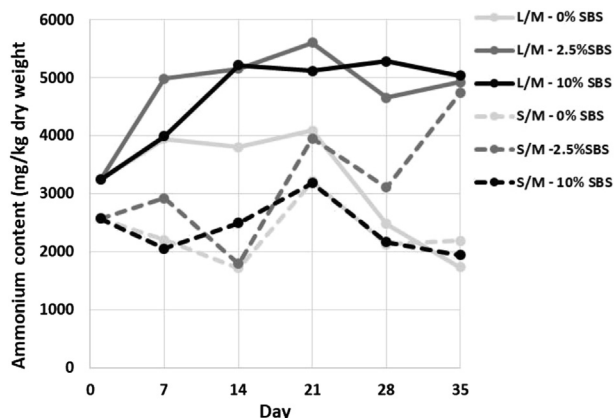
<sup>a-c</sup>Means within columns not sharing common suffixes are significantly different at the 5% level of probability. Abbreviation: SBS, sodium bisulfate.

performed to evaluate the effect by using Tukey HSD in JMP, version 14 (SAS Institute Inc., Cary, NC).

## RESULT AND DISCUSSION

### Physical and Chemical Properties of the Litter/Manure and Shavings/Manure Mixtures

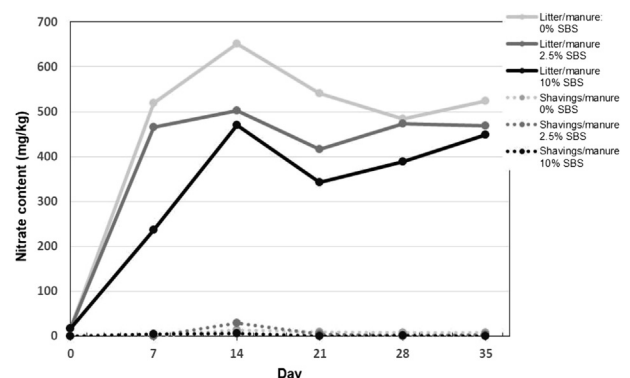
Shavings and litter are good water absorbents and buffer materials that could reduce skin lesion of broilers. The moisture contents of fresh manure from 3-week of age broiler was higher than those of pine shaving and built-up litter (Table 1). In this study, the moisture contents of the different mixtures over the course of the experiment are illustrated in Figure 1. The water content in the initial mixtures evaporated into the air and the moisture level dropped continuously over the first 2 wk. Then, additional water was added to bring the moisture level back up to a level of about 20 to 25%, a level more in line what is found in commercial poultry litter (Miles et al., 2011). The litter moisture in commercial broiler operations could change from 10% in summer with chicks to 50% in winter with 6-week-old broilers (Miles et al., 2011). The 6 mixtures had similar moisture levels at each time point, and no significant difference was found among them because they shared the same environment (Table 2).



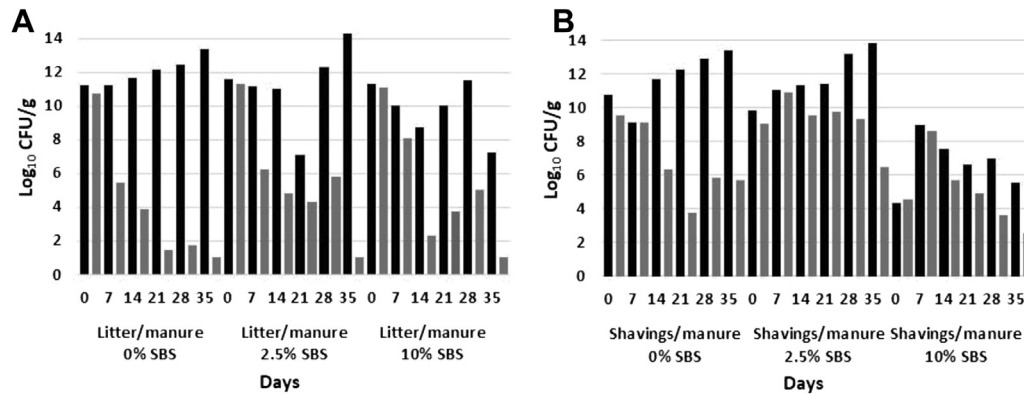
**Figure 3.** Ammonium content of litter/manure (L/M) and shavings manure (S/M) samples with and without sodium bisulfate (SBS) supplementation.

The pH of the different mixtures during the 5-week experimental period is shown in Figure 2 and Table 3. The pH of the litter/manure mixture without SBS increased from an initial level of 6.4 to around 7.2 over the course of 2 wk, then remained stable. Although litter pH values reported in the literature are not easy to compare as litter type, flock status, and litter age vary, and the pH values obtained for the SBS-free (0% SBS) litter samples are in line with those reported for some studies (Li et al., 2013; Crippen et al., 2016; Sahoo et al., 2017; Payne et al., 2019); however, higher pH values were reported in other studies. For example, Hunolt et al. (2015) measured pH values between 8.06 and 8.71, similar to those reported by Williams et al. (2012). It is likely that the initial low pH (~6.0) of fresh manure and the volatilization of the ammonia produced in the unamended samples kept the pH at the lower level. Addition of 2.5 and 10% SBS resulted in initial pH levels of 4.8 and 2.5, respectively. The pH levels on day 35 were 6.6 and 3.37, respectively. The initial pH levels of the shavings/manure mixtures were lower than those of the corresponding litter/manure mixtures and remained so during the 5-week trial presumably because of lower buffering capacity of fresh shavings (Table 3).

Ammonium levels were highest in the litter/manure samples supplemented with SBS (Figure 3). As expected, the lower pH of these samples resulted in the retention of ammonia nitrogen as ammonium salt. At the pH levels observed for the unamended litter/manure



**Figure 4.** Nitrate content of litter/manure and shavings/manure samples with and without supplementation with sodium bisulfate (SBS).



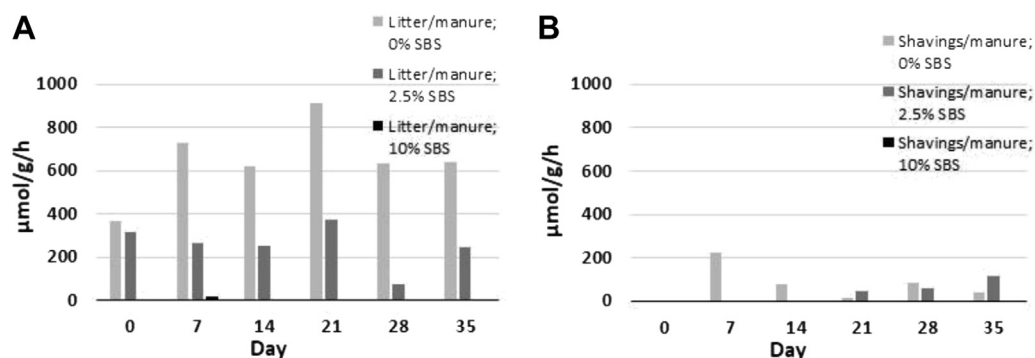
**Figure 5.** A. Total aerobic counts (black bars) and coliform counts (gray bars) in litter/manure samples with and without sodium bisulfate (SBS) addition. B. Corresponding counts in shavings/manure samples. Data are CFU per g of dry litter.

sample, some of the nitrogen will exist as ammonia, which can escape into the atmosphere, resulting in lower ammonium concentrations. The ammonium contents of the shavings/manure samples were generally lower than those of the litter/manure samples. It is possible that the similar levels of ammonium in the unamended sample and that amended with 10% SBS are because of the very low pH of the amended sample preventing the generation of ammonia once SBS had been dispersed throughout the matrix. The ammonium level of the unamended sample could have been low because of the release of ammonia into the atmosphere. The higher levels of ammonium present in the shavings/litter samples could have been because of ammonia production and retention at the pH of the sample.

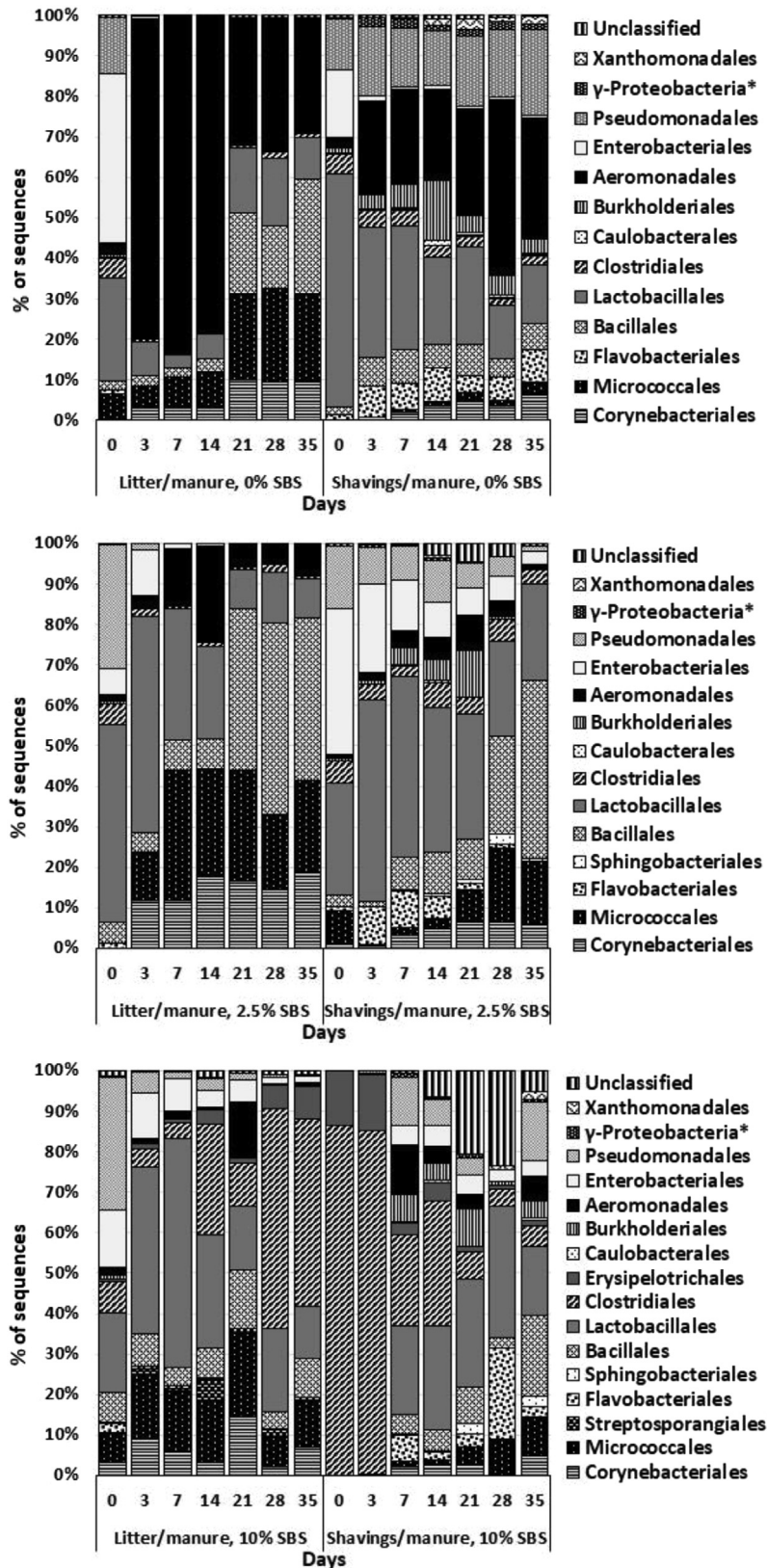
Noticeable differences between the litter/manure and the shavings/manure samples were observed for their nitrate content (Figure 4). The litter manure samples produced nitrate regardless of their SBS content, indicating the presence of ammonia and nitrite oxidizing bacteria and/or little removal of nitrate by denitrification. In contrast, the shavings/manure samples accumulated only low amounts of nitrate. It appears that components of the nitrogen-metabolizing microbial consortia were not present in the shavings or manure. Alternatively, nitrate metabolism could have been high in these samples.

### Total Aerobic and Coliform Counts

The counts obtained are depicted in Figure 5. The low plate counts for the shavings/manure samples supplemented with 10% of SBS on days 0 and 3 (Figure 5B) were likely caused by the low pH encountered during the extraction procedure (stomaching and filtration). Therefore, it was decided to adjust the pH of all samples to at least 6.5 immediately after the addition of PBS. Consequently, the total aerobic counts in the shavings/manure samples with 10% SBS increased from around  $10^4$  cfu/g of dry litter to  $10^9$  cfu/g of dry litter for the sample taken on day 7. A similar increase was observed with the coliform counts (Figure 5B). When looking at the total aerobic counts and the coliform counts for the unamended litter/manure samples (Figure 5A), it is obvious that the total aerobic counts showed an increasing trend, whereas the coliform counts decreased over time. The counts for the unamended shavings/manure samples followed a similar trend, but with the coliform counts remaining at a higher level (Figure 5B). The trends in the corresponding counts for the amended samples are varied. In the presence of 2.5% amendment, coliform counts remained relatively stable after 7 d for both litter/manure and shavings/manure samples. In contrast, the litter/manure samples amended with 10% SBS showed a decline, then an



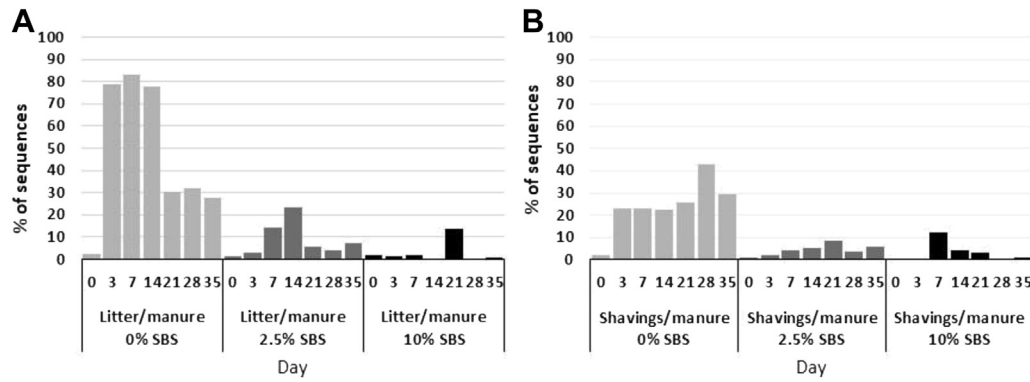
**Figure 6.** A. Urease activity ( $\mu\text{mol}$  ammonia produced per g of dry sample per hour) in litter/manure samples with and without sodium bisulfate addition. B. Corresponding values for shavings/manure samples. Abbreviation: SBS, sodium bisulfate.



**Figure 7.** Percent of sequences belonging to taxonomic orders from litter/manure and shavings/manure samples with and without sodium bisulfate (SBS) addition. Top panel: samples without SBS; center panel: samples with 2.5% SBS; bottom panel: samples with 10% SBS. \*sequences identified as belonging to the  $\gamma$ -Proteobacteria, but not classifiable further.

increase in coliform counts. The shavings/manure samples showed counts that declined continuously over time. In general, however, coliform counts on

corresponding days were higher in the amended samples. For example, on day 21, coliform counts in the litter/manure and shavings/manure samples amended with



**Figure 8.** A. Percent of sequences derived from litter/manure samples with and without sodium bisulfate addition that represent the genus *Oceanisphaera*. B. Corresponding percentages for the shavings/manure samples. Abbreviation: SBS, sodium bisulfate.

2.5% SBS were about  $10^4$  and  $10^9$  cfu/g of dry mass, respectively. Similarly, coliform counts in the corresponding samples amended with 10% SBS were approximately  $10^4$  and  $10^5$  cfu/g of dry matter, respectively. In contrast, the unamended samples showed counts of  $10^1$  and  $3.5 \times 10^3$  per g of dry matter. The reason why the coliform counts were lowest in the litter/manure sample without SBS amendments is perhaps an inhibitory effect of  $\text{NH}_3$  on these types of bacteria.

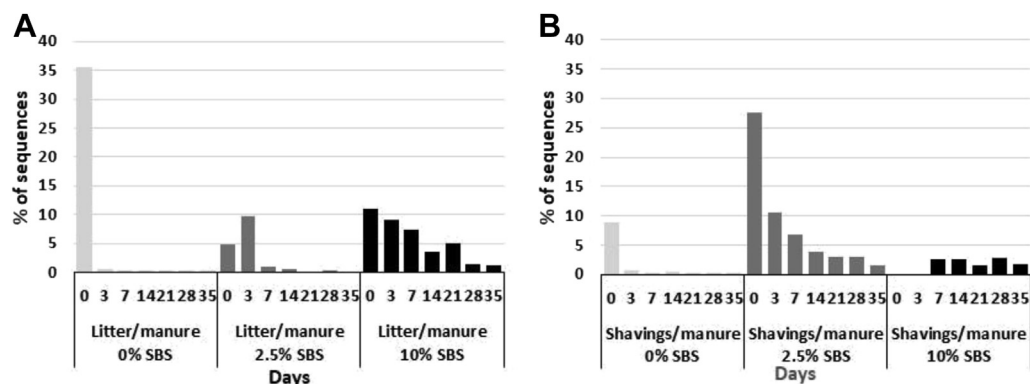
### Urease Activity

The uric acid excreted by the birds is metabolized via several intermediates to urea that is finally broken down into carbon dioxide and ammonia by the action of urease. This enzyme is inducible by urea in some bacteria, but by general nitrogen deficiency in others, or it can even be produced constitutively (Mobley and Hausinger, 1989). In the present study, the highest urease activities were observed in unamended litter/manure and shavings/manure, although the numerical activities were considerably lower in the shavings/manure samples (Figure 6). Samples amended with 2.5% SBS also showed urease activity, but no activity was detected in the litter sample receiving 10% of the acidic compound. The absence of measurable urease activity in these samples could be because of suppression of uric acid-degrading

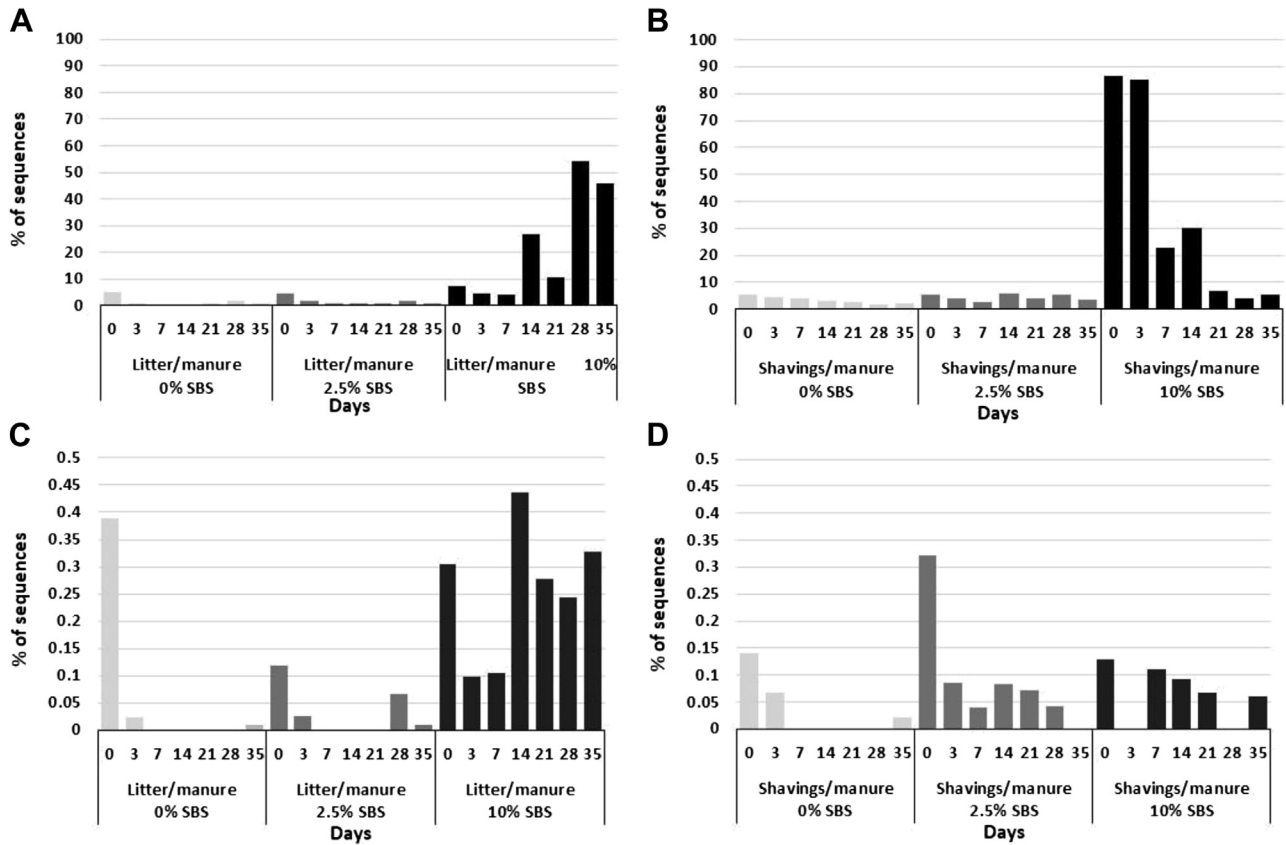
and urease-producing bacteria or the suppression of urease synthesis as ammonia under low pH conditions is available as  $\text{NH}_4^+$  and does not escape into the atmosphere as  $\text{NH}_3$ .

### Microbial Population Analysis

Ribosomal RNA gene sequence-based analysis of microbial populations in litter have previously been carried out on a few of occasions. For example, Lu et al. (2003) constructed a clone library of PCR-amplified 16S rRNA genes present in DNA extracted from chicken litter samples and sequenced the ribosomal DNA inserts. Most of the sequences originated from low-GC gram positive bacteria, with high-GC gram positive and proteobacteria making up the rest. While that study was able to look at a total of only 340 sequences, the advent of high-throughput sequences allowed Dumas et al. (2011) to analyze several thousand sequences from 8 litter samples with the goal of finding a link between the microbial communities present at a location and the occurrence of gangrenous dermatitis. The number of sequences originating from poultry litter samples was further increased in a study by Locatelli et al. (2017) who analyzed a total of over 200,000 sequences, with more than 7,000 sequences originating from each litter sample. In the present study, a total of 46 samples representing the 3 treatments for 2 types of



**Figure 9.** A. Percent of sequences in litter/manure samples with and without sodium bisulfate (SBS) amendment representing the genus *Escherichia*. B. Corresponding percentages for shavings/manure samples.

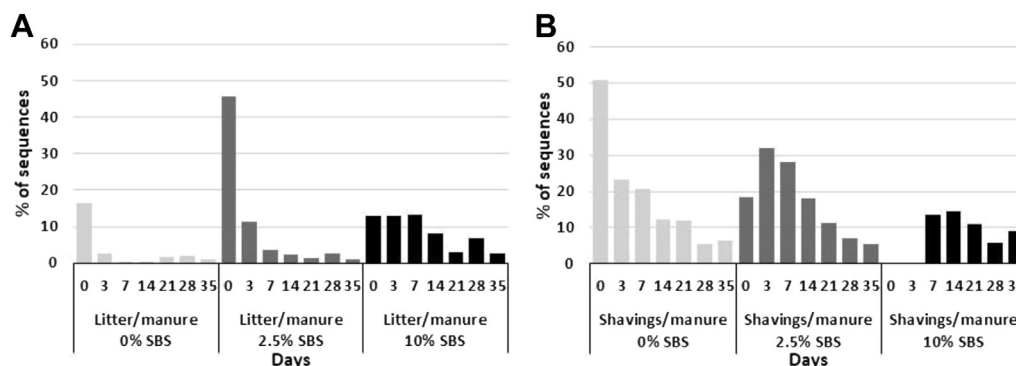


**Figure 10.** A. Percent of sequences in litter/manure samples with and without sodium bisulfate amendment representing Clostridiales. B. Corresponding percentages for shavings/manure samples. C. Percent of sequences in litter/manure samples representing *Clostridium perfringens*. D. Corresponding percentages for shavings/manure samples. Abbreviation: SBS, sodium bisulfate.

litter over a 5-week period were analyzed using Illumina 454 sequencing. A total of 405,447 sequences were obtained that could be analyzed. The numbers of sequences obtained from each sample ranged from 6,955 to 13,606 with an average of 9,653. The percent of sequences representing the different taxonomic groups were graphically displayed and visually searched for differences in the occurrence of sequences between treatments and over time.

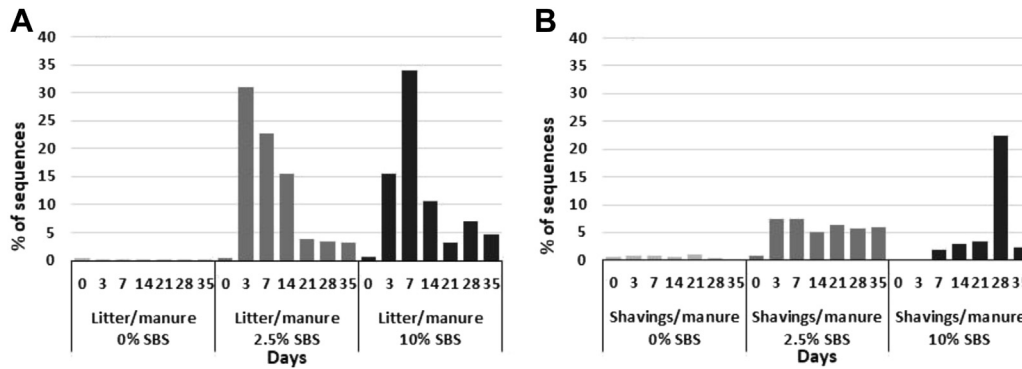
Figure 7 displays the percentages (>1%) of sequences belonging to different bacterial orders for litter/manure and shavings manure samples with and without the addition of SBS. The most obvious difference between

the samples with and without SBS is the strong presence of sequences belonging to the order *Aeromonadales* in the unamended samples. Another difference is the disappearance of sequences belonging to the *Enterobacteriaceae* from the SBS-free samples by day 3, but their persistence, albeit at lower percentages in the samples with SBS throughout the trial. Sequences from the order *Lactobacillales* were present in all samples. The percentage of sequences belonging to the order *Clostridiales* was highest in the samples with 10% SBS and lowest in the samples without SBS. Sequences representing the orders *Caulobacterales* and *Xanthomonadales* were only observed for shavings/manure samples.



**Figure 11.** A. Percent of sequences representing *Lactobacillus* sp. in litter/manure samples with and without the addition of sodium bisulfite (SBS). B. Corresponding percentages for shavings/manure samples.



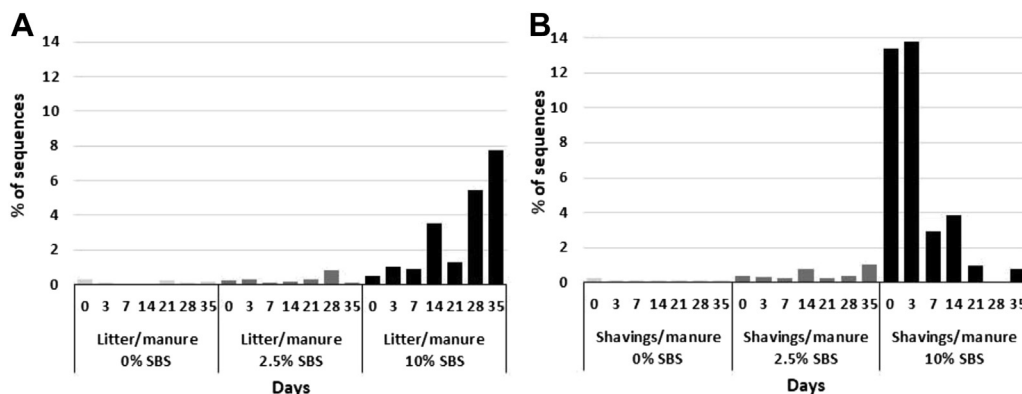


**Figure 12.** A. Percent of sequences representing *Weisella* sp. in litter/manure samples with and without amendments of sodium bisulfate (SBS). B. Corresponding percentages for shavings/manure samples.

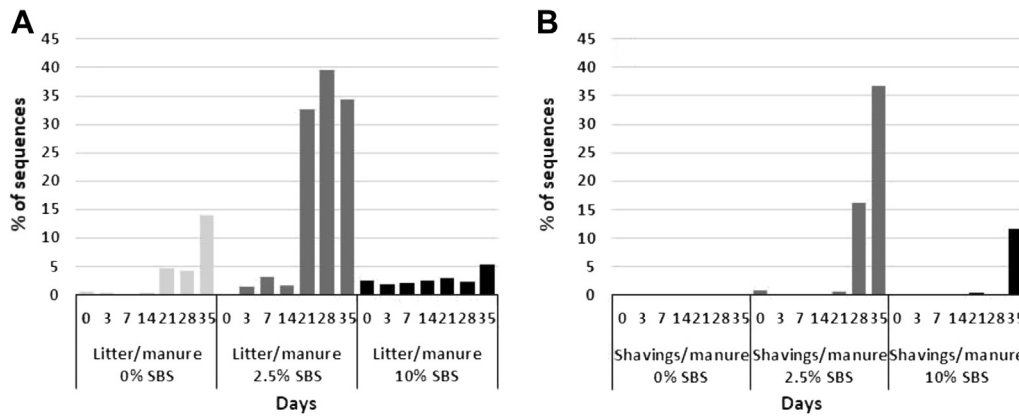
When analyzing the presence of sequences at the genus level, it is obvious that *Aeromonadales* sequences are primarily because of the presence of sequences from the genus *Oceanisphaera*. At the onset of the experiment, in all samples, about 1% or less of sequences belonged to this genus. This percentage increased to about 80% by day 3 in the untreated litter/manure sample and about 25% in the treated samples (Figure 8). The significance of the presence of members of the genus *Oceanisphaera* in the litter/manure and shavings/manure samples is not known. Until recently, all members of the genus *Oceanisphaera* had been isolated from marine sources (Liu et al., 2017); however, a new species, *Oceanisphaera avium*, was described that was isolated from the gut of the vulture, *Aegyptius monachus* (Sung et al., 2018). Members of the genus *Oceanisphaera* are halophilic and show optimal growth around pH 8. To our knowledge, sequences from members of the genus *Oceanisphaera* have not previously been observed in poultry litter or poultry intestinal samples. It is possible that members of this genus were present in the litter or manure employed in this study because of some unknown, unique circumstance and that they proliferated at a high rate once  $\text{NH}_3$  production and pH of the sample increased, but it is also possible that this genus is present in litter environments more broadly and has previously not been observed because of the relatively low numbers of litter samples that have been studied.

The majority of sequences from the order *Enterobacteriales* belonged to the genus *Escherichia*. As seen in Figure 9, the initial high percentage of sequences from the genus *Escherichia* of approximately 40% decreased within 3 d to below 1% in the unamended litter/manure sample (Figure 9A). A similar drop was also seen with the shavings/manure sample (Figure 9B). In contrast, *Escherichia* sp. persisted or declined slowly over the 5-week period of the study in the litter samples amended with 10% sodium sulfate, but especially in the shavings/manure sample amended with 2.5% sodium sulfate. These data suggest that higher  $\text{NH}_3$  levels caused suppression of *Escherichia* sp., whereas the reduced pH and lower free ammonia levels allowed persistence of members of this genus.

This outcome raises the question if other members of the *Enterobacteriales*, such as *Salmonella*, would have been affected similarly. The impact of litter amendments on the survival of pathogens such as *Salmonella enterica* in poultry litter is obviously of practical importance, and as described in the Introduction, has been the topic of several earlier studies. Because no *Salmonella* sequences were found, the present study cannot directly address the impact of the treatments on this pathogen; however, it is possible that, like *Escherichia*, *Salmonella* would also have fared worse in untreated litter as observed by Williams et al. (2012). The plating data (Figure 5) agree with the sequencing data in that it showed a faster



**Figure 13.** A. Percent of sequences retrieved from litter/manure with and without sodium bisulfate amendment representing *Turicibacter sanguinis*. B. Corresponding percentages for shavings/manure samples. Abbreviation: SBS, sodium bisulfate.



**Figure 14.** A. Percent of sequences in litter/manure with and without sodium bisulfate (SBS) amendment representing *Staphylococcus* sp. B. Corresponding percentages for shavings/manure samples.

decline of coliforms over time in unamended than in the SBS-amended samples.

Members of the order *Clostridiales* are common intestinal bacteria, but some are of concern to poultry health as they are the causative agents of necrotic enteritis (Fasina et al., 2016). The 16S rRNA gene sequence analysis of the litter samples indicated that, except for the samples treated with 10% SBS, members of the *Clostridiales* made up only a small part of the bacterial population (Figures 10A, 10B).

Overall, samples with the high SBS amendment showed higher percentages of *Clostridiales* sequences. Whether these sequences stemmed from vegetative cells or spores is not known. It is likely that the high percentages of *Clostridiales* sequences for the shavings/manure sample amended with 10% SBS on days 0 and 3 were because of the resistance of spores to the harsh pH conditions during sample extraction. *Clostridium perfringens* sequences were present in low numbers but were more common in samples with 10% SBS (Figures 10B, 10C).

16S rRNA gene sequences representing the order *Lactobacillales* were found in all samples (Figure 7). Sequences belonging to the genus *Lactobacillus* sp. were most common at the onset of the experiment, likely because of relatively fresh manure having been added to litter or shavings (Figure 11). The absence of sequences from this order for days 0 and 3 in the shavings/manure sample (Figure 11B) is likely due to the extraction procedure having destroyed these bacteria as they were exposed to an extreme pH. In general, the *Lactobacillus* sequence percentages declined in the litter/manure samples regardless of the treatment, although higher percentages were seen in the amended samples, possibly because of the lower pH, a condition that provided acid-tolerant species with an advantage. The genus *Weissella* was represented by only a small number of sequences at the beginning of the experiment (Figure 12). The numbers stayed low for unamended litter/manure but increased by day 3 in the other 2 types of samples. Although a few species of this genus are opportunistic pathogens (Abriouel et al. 2015), *Weissella* species are likely of no concern in litter and their higher numbers compared with those of the genus

*Lactobacillus* suggests better adaptation to the conditions present in the treated samples.

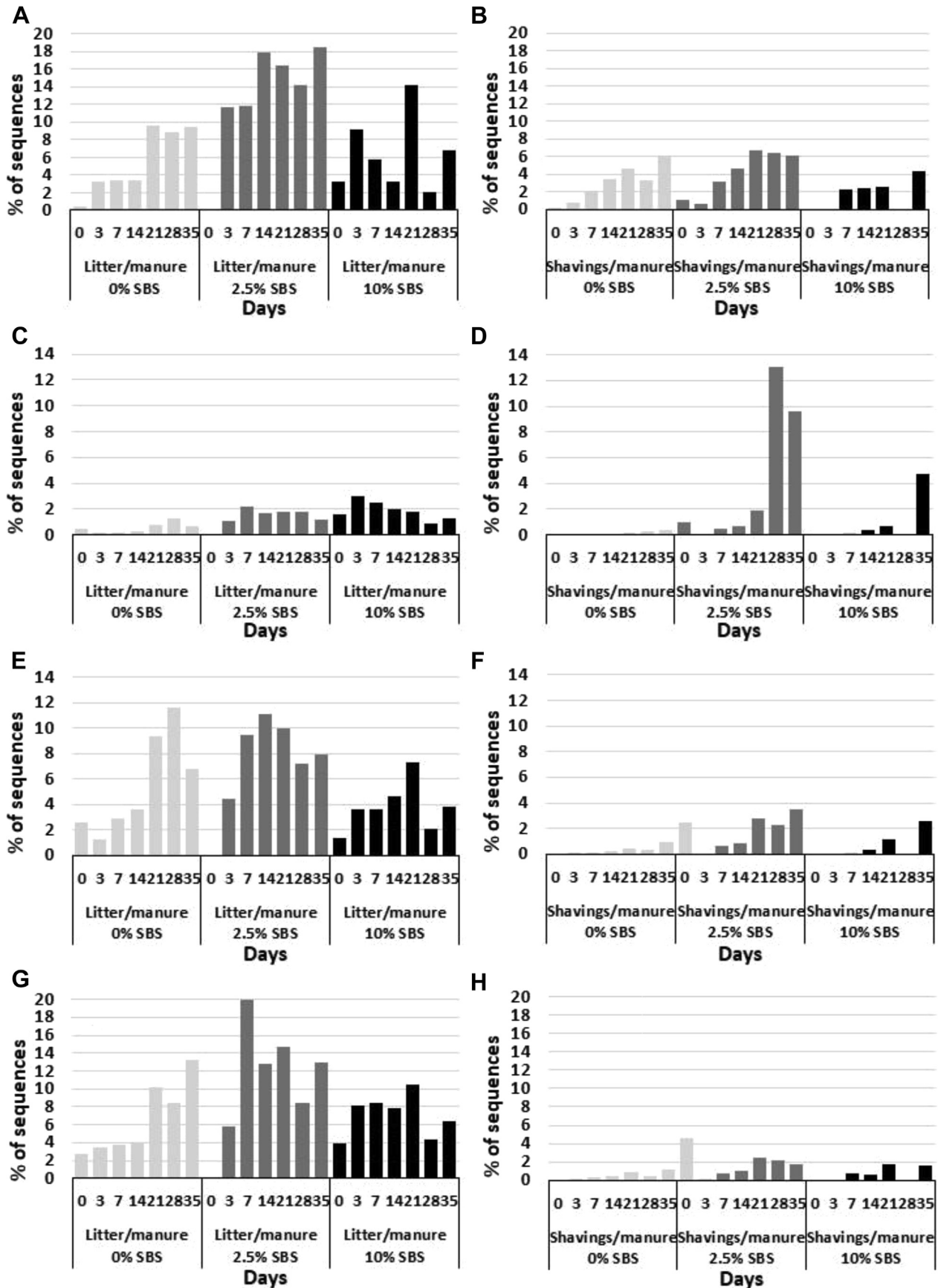
The order *Erysipelotrichales* was represented mostly by sequences belonging to the genus *Turicibacter*. As seen in Figure 13, these sequences were preferentially found in the samples amended with 10% SBS. The reasons for the occurrence of these sequences and the consequences of the presence of these types of bacteria in litter are not known. Data on this genus are scarce, and the only species described so far, *Turicibacter sanguinis*, was isolated from blood of a patient (Bosshard et al., 2002).

Members of the genus *Staphylococcus* have been observed in culture-based (Roberts et al., 2013; Williams and Macklin, 2013) and sequencing-based litter studies (Dumas et al., 2011). The current sequence data indicate that members of this family fared best in litter/manure and shavings/manure when amended with 2.5% SBS, and their sequences reached over 40% of the total sequences obtained from samples on a particular day (Figure 14).

The order *Corynebacteriales* was represented by sequences from the genus *Corynebacterium*, particularly *Corynebacterium stationis*. A higher percentage of these sequences was found in samples amended with 2.5% SBS than in unamended samples and those amended with 10% of the acidifier (Figures 15A, 15B). *Brevibacterium* sp., in the order *Micrococcales*, also appears to have benefitted from a more acidified environment as sequence percentages were generally higher in amended samples (Figure 15C, 15D). Among the Brevibacteria, *Brevibacterium avium* is considered pathogenic as it is associated with bumble foot lesions (Pascual and Collins, 1999), and increased survival or proliferation by this bacterium could be a concern.

The other 2 genera from the order *Micrococcales* were represented by sequences from *Brachybacterium* and *Arthrobacter*. Sequences from these species were present in all samples over the 5-week period (Figures 15E–H), with a slightly higher percentage in samples amended with 2.5% SBS. *Brachybacterium* and *Arthrobacter* sp. were previously observed in litter (Dumas et al., 2011) but have not been implicated in disease.

Overall, the sequence-based population data indicate that litter treatments had no discernable effect on



**Figure 15.** A. Percent sequences attributed to *Corynebacterium stationis* in litter/manure samples. B. Corresponding percentages for sequences from shavings/manure samples. C. Percent sequences representing *Brevibacterium avium* from litter/manure samples. D. Corresponding percentages for sequences from shavings/manure samples. E. Percent sequences representing the genus *Brachybacterium* from litter/manure samples. F. Corresponding percentages from shavings/manure samples. G. Percent of sequences belonging to the genus *Arthrobacter* from litter/manure samples. H. Corresponding percentages for sequences from shavings/manure samples. Abbreviation: SBS, sodium bisulfate.

some groups of bacteria, enhanced survival of some groups of bacteria and suppressed others. Some data suggest that groups of bacteria of concern to poultry or human health could be enriched in litter amended with SBS, but additional studies will need to be done to determine which observations made with the present study are common to acidic litter treatments and which ones are due to unique factors.

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## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.08.013>.

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