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Influence of Manure Application Techniques on the Microbial Content of Grass and Grass Silage

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ABSTRACT

Field observations of visible dry manure residues in organically fertilised grassland, especially in drought areas in Bavaria (Germany), raised questions about whether different manure application methods influence the microbial composition in grass and in silages. Therefore, the aim of this study was to investigate if and to which extent three manure application methods (broadcast, trailing shoe and disc injector) and a control (mineral fertiliser) influence the microbial quality of grass and grass silage. The following samples were taken in two trial years (2020–2021): Soil ($n = 16$), manure ($n = 10$), wilted-chopped grass ($n = 96$) and grass silage samples ($n = 80$). The laboratory methods used were cultivation and qPCR. The comparison between the test groups showed no significant difference in the number of aerobic bacteria, lactic acid bacteria, *Lactobacillus* spp., *Enterobacteriaceae*, *E. coli* and yeasts in all sample types. The Clostridia load in soil and grass before fertilisation was similar in all test groups. After fertilisation, grass samples from plots fertilised with the disc injector method had statistically significantly lower Clostridia (2.6 $\log_{10}$ cfu/g) than samples from the trailing shoe (3.3 $\log_{10}$) and the broadcast (3.2 $\log_{10}$) but higher than the control group (1.7 $\log_{10}$) ($p < 0.05$). Clostridia counts in silages were between 3.7 and 3.9 $\log_{10}$ for the manure treatments and 3.3 $\log_{10}$ for the control. Except for the Clostridia levels in the grass, the results of this study indicate that the grass and silage from the three manure application methods were of similar microbial quality.

1 | Introduction

Grass silage is widely used as ruminant fodder around the world, particularly in Europe and North America (Ávila and Carvalho 2020; Driehuis and Elferink 2000). The main objective of silage production is to preserve forage for periods of feed scarcity due to seasonal fluctuations, which commonly happen during winter in the temperate regions or dry seasons in the countries in tropical climates (Driehuis and Elferink 2000). This is particularly relevant in countries with intensive farming systems and long winter periods like Germany, Denmark and the Netherlands, where ensiled grass represents over 90% of preserved forage (Wilkinson et al. 1996).

Since livestock manure represents a valuable source of nitrogen, phosphorus and potassium, as well as numerous micronutrients, it is used as a fertiliser to promote the growth of grass and crops. In addition, spreading liquid manure on grassland

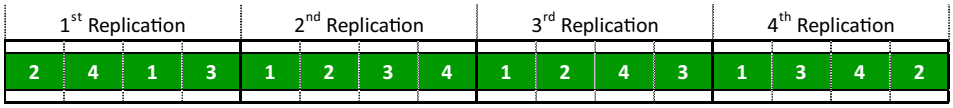

FIGURE 1 | Blocks (1st–4th replications) and plots (1–4) for each fertilising method: 1 = mineral fertiliser (calcium ammonium nitrate) as control, 2 = broadcast (splash plate), 3 = trailing shoe, and 4 = disc injector.

is more cost-effective than inorganic fertilisers and contributes to the concept of a circular economy. However, there is also a risk of spreading pathogens or spoilage microorganisms through manure or slurry (Östling and Lindgren 1991; Manitoba 2015; Rammer et al. 1997). In general, conventional surface-broadcast manure/slurry application techniques like the splash plate applicator are fast and low-cost manure distribution methods (Webb et al. 2010). Downsides of splash plate application reported in numerous studies include inaccurate distribution, nitrogen losses and an increase in odour and ammonia (NH_3) emissions, as well as chemical burns of plants. Consequently, this manure application method can have a negative impact on the growth of grass. In addition, residues of manure on grasses can also be associated with an increase in the number of undesirable microorganisms on the plants (Häni et al. 2016; Huijsmans et al. 2001; Misselbrook et al. 2002; Müller 1993; Rzeźnik and Mielcarek-Bocheńska 2020; Webb et al. 2010).

The National Emission Ceilings (NEC) Directive, in accordance with Directive (EU) 2016/2284 2016, sets targets to reduce specific air pollutants in the European Union Member States. In Germany, for example, NH_3 emissions were required to be reduced by 5% by 2020, and must be reduced by 29% by 2030 compared to the reference year 2005. Approximately 95% of NH_3 emissions in Germany currently arise from agriculture, for example, from manure, slurry and fermentation residues from biogas plants (LfL 2020). Therefore, the agricultural sector is under particular pressure to achieve the NH_3 emission targets, and as a result, the competent authority in Germany has decided that from 2025 onwards, manure fertilisation of grassland and multi-cut forage crops must be carried out using near-surface or subsurface application methods such as trailing shoes and injecting instead of surface-broadcast spreading methods (LfL 2020).

It is possible that manure can adhere to the grass when fertilising grassland. In general, the risk of manure residues on grass is lower when manure is applied close to the ground (Huijsmans et al. 2001). However, in some drought-prone areas in Bavaria (Germany), especially in years with long summer drought and low precipitation between fertilisation and harvest time, such as in 2018, there were field observations of visible dry manure residues in grass when near-surface or ground-level manure applications were applied (LfL 2023). There is limited data on the influence of these dry manure residues on the hygiene of the preserved forage, such as silage. Results of similar studies from regions with more precipitation, like Switzerland (Wyss et al. 2017) and Austria (Pöllinger et al. 2018), cannot be transferred to the dry locations.

Therefore, the aim of this study was to investigate the extent to which manure application methods influence the microbial count of harvested and wilted-chopped grass prior to ensiling as well as that of the resulting silage. All experiments were conducted in a drought-prone region of Lower Franconia in the state of Bavaria, Germany. The manure application techniques in this study were near-surface and sub-surface methods (trailing shoe and disc injector, respectively) and a conventional surface-broadcast spreading method (with a splash plate). Plots treated with mineral (inorganic) fertiliser served as a control. As a readout, aerobic plate counts, lactic acid bacteria, *Lactobacillus* spp., *Enterobacteriaceae*, *E. coli*, *Clostridium* spp. and yeasts were determined.

2 | Materials and Methods

2.1 | Field Plots and Fertilising Techniques

The field experiments were conducted in the district of Lower Franconia, Bavaria, Germany (altitude 284 m above sea level, average annual rainfall of 537 mm and temperature of 10°C). The grassland consisted mainly of meadow foxtail (*Alopecurus pratensis*). Agricultural maintenance and manure application on the trial plots were carried out by the Institute for Organic Farming, Soil and Resource Management of the Bavarian State Research Centre for Agriculture (LfL, Germany). The trial area has not been fertilised since summer 2019, and the last grass harvest took place in autumn 2019.

The present trial started in March 2020. For this purpose, the experimental field (576 m^2) was separated into four blocks (144 m^2 each). Each block represented a replicate and was divided into four plots (36 m^2 each). Three manure spreading methods and a control group were applied to the plots in a randomised fashion (see Figure 1, Dorn-In et al. 2025):

- Broadcast with splash plate (self-built model by Bavarian State Research Centre of Agriculture, Germany) with 3 m working width
- Trailing shoe (Bomech Farmer, the Netherlands) with 3 m working width
- Disc injector (Veenhuis Euroject, the Netherlands) with 3.04 m working width
- Control plots were fertilised with manually applied inorganic mineral fertiliser (calcium ammonium nitrate, CAN)

The amount of manure applied per plot was calculated based on the nitrogen content of the manure using the Kjeldahl method, with a target value of 170 kg of nitrogen per hectare and year. The first manure application took place in March 2020.

In terms of weather conditions, the first year of the trial (2020) began with cool temperatures and low rainfall in spring that led

to delayed growth of the grass. The summer season was characterised by long periods of drought, followed by humid autumn and unusually warm weather conditions in November that enabled a late third harvest in November 2020. In the second trial year, 2021, there were copious rainfalls in spring, which were unusual for the region. The early summer was then characterised by changeable weather periods with warm temperatures and numerous rainfalls.

2.2 | Field Samples

Four types of samples were analysed, namely (1) soil; (2) manure; (3) wilted-chopped grass; and (4) grass silage. Although meadow foxtail is the predominant species in the trial field, the terms 'grass' and 'grass silage' used in this study are not exclusive to this species, as the composition also includes other plant species in smaller quantities.

Soil ($n = 16$) and wilted-chopped grass samples (G0, $n = 16$) were taken before the beginning of the trial. During the trial period (2020–2021), there were five sampling times for each sample type, namely manure (M1–M5, $n = 10$), wilted-chopped grass (G1–G5, $n = 80$) and grass silage (S1–S5, $n = 80$). Table 1 shows the exact date of sampling for each sample type. The manure came from a dairy farm in Lower Franconia, Bavaria (Germany). It was stored in a covered manure pit until use, 5 months for the first (M1) and 17 months for the last (M5) fertilisation. Manure was applied immediately after harvesting grass G0, G1 and G2, and 3 days after cutting grass G4, respectively. The intervals between manure application and grass harvest were typically 5–7 weeks, except for grass G3, where grass harvesting did not take place until 18 weeks after manure application due to weather conditions.

TABLE 1 | Sampling date of each sample type.

	Sample			
Sampling date	**Soil[a]**	**Manure**	**Grass[a]**	**Silage**
16 March 2020	Soil	M1	G0	—
05 May 2020	—	M2	G1	—
23 June 2020	—	M3	G2	—
10 August 2020	—	—	—	S1
21 September 2020	—	—	—	S2
04 November 2020	—	—	G3	—
16 February 2021	—	—	—	S3
06 April 2021	—	M4	—	—
15 May 2021	—	—	G4	—
19 May 2021	—	M5	—	—
03 July 2021	—	—	G5	—
16 August 2021	—	—	—	S4
04 October 2021	—	—	—	S5

[a]At the same sampling date, soil and grass samples were collected before the manure was distributed on the experimental field plots. After harvesting, grass was wilted and then chopped (preparing for ensiling), and then chopped grass was sampled for the investigation.

The details of the sampling procedures for soil, manure, grass and silage samples were described in a previous publication (Dorn-In et al. 2025). In brief, soil samples were taken from 20 locations in each plot, then mixed and 200 g of the mixed soil was placed in a sterile plastic bag. At each sampling time, the liquid manure was filled from the tank of the slurry spreader into a 2-L bottle before being spread on the experimental field. Approximately 20 kg of fresh grass was harvested from each plot, wilted and then chopped to a length of 2–6 cm. Subsequently, 500 g of the wilted-chopped grass samples were collected in a sterile plastic bag. The silage samples (S1–S5) were prepared accordingly from the grass samples G1–G5 of each plot. The wilted-chopped grass from each plot was separately subjected to a laboratory-scale ensiling process with three technical replicates. It was pressed into a 1.75-L glass jar, sealed with a lid to prevent air permeability and stored at 25°C ± 1°C for 90 days. After ensiling, 100 g of each of the three replicates were manually mixed and analysed.

2.3 | Microbiological Examination

Live microorganisms have an impact on the safety and quality of food and feed, which is why culture methods are considered the gold standard for assessing the quality of these sample types (Foddai and Grant 2020). The preparation of the samples and the cultivation methods for the respective microbial groups were carried out according to the methods provided by the International Organisation for Standardisation or adapted from the publication. Ten gram of each sample (soil, manure, grass or grass silage) and 90 mL of buffered sodium chloride peptone solution (BSP, Merck) were mixed for 60 s in a bag mixer (Bagmixer, Interscience). A tenfold serial dilution in BSP up to 10^{-6} was performed for all sample types. Subsequently, 100 μL of the three appropriate dilutions of each sample type were spread on the respective agar.

The following agars and incubation conditions were applied: Plate Count agar (PCA; Merck) for total aerobic plate count (APC, aerobe, 30°C for 72 h), Violet Red Bile Dextrose agar (VRBD, Merck) for *Enterobacteriaceae* (anaerobe, 30°C for 48 h) and Rapid *E. coli* agar (REC; Bio-Rad) for *E. coli* (aerobe, 44°C for 24 h). DeMan Rogosa Sharpe agar (MRS; Sifin) was used for lactic acid bacteria (LAB, anaerobe, 30°C for 72 h), while cultivation of *Lactobacillus* spp. (anaerobe, 37°C for 72 h) was performed using *Lactobacillus* Anaerobic MRS with Vancomycin and Bromocresol green agar (LAMVAB; Bromocresol green and MRS agar: Sigma, Cysteine HCl and MRS broth: Merck, Vancomycin: PanReac AppliChem) (Hartemink et al. 1997). Yeasts were cultured on Malt Extract agar (Merck) with Novobiocin (Roth) (MEA+) and aerobically incubated at 25°C for 4–5 days.

Schaedler agar (SCHA; Thermo Scientific, Oxoid) is recommended for the cultivation of anaerobic or facultative anaerobic bacteria. In the present study, it was used to culture spore-forming bacteria from manure (M1–M5), grass (G1–G5) and grass silage (S1–S5). One millilitre of the sample suspension

(dilution 10^{-2} to 10^{-4}) in BSP was heated at 80°C for 10 min in a thermomixer (Eppendorf) to inactivate vegetative cells of other accompanying microbiota. Subsequently, 100 μL of the heat-treated suspension was spread on SCHA agar and anaerobically incubated at 37°C for 72 h. In addition to *Clostridium* spp., other spore-forming, facultative anaerobic bacteria such as *Bacillus* spp. and *Paenibacillus* spp. can survive heat treatment and grow on SCHA agar. The latter two genera were ubiquitous in manure, soil and grass. According to the investigation at the beginning of this study, the colony morphology of the genera *Clostridium*, *Bacillus* and *Paenibacillus* on SCHA is very similar and cannot be distinguished. Therefore, the colonies grown on SCHA agar were not counted but only analysed for species identification using MALDI-TOF MS. The AnaeroGen (Thermo Fisher Scientific, Oxoid) was used to create anaerobic conditions in the bags or jars containing VRBD, MRS, LAMVAB and SCHA plates.

2.4 | Molecular Biological Examination

The bacterial species of the genus *Clostridium* spp. exhibit a wide variety of characteristics; thus there are no suitable selective culture media that could be applied to all of them. Therefore, colonies growing on used media need to be confirmed by further laboratory methods such as MALDI-TOF MS and DNA sequencing. For this reason, the quantification of these bacteria using the culture method is usually difficult and time-consuming (Dorn-In et al. 2025). To overcome this challenge, a specific quantitative PCR (qPCR) for Clostridia was developed and applied to the investigated samples (Dorn-In et al. 2025). The ISOLATE II Genomic DNA Kit (Meridian Bioscience, BioCat GmbH) was used to extract DNA from manure, grass and silage samples. The MoBio PowerSoil DNA-Isolation Kit (Qiagen) was used to extract DNA from soil samples. The primer pair Cl346-F (5′-TATTGCACAATGGGGGAAACC-3′) and Cl642-R (5′-CCTCTCCTGCACTCTAGA-3′) was used to amplify the 16S rRNA gene of Clostridia. The fragment size of the PCR amplicons is 278 bp. The details of DNA extraction methods and qPCR for Clostridia are described in detail in a previous publication (Dorn-In et al. 2025).

2.5 | MALDI-TOF MS

Lactobacillus spp. are the preferred bacteria for the ensiling process in order to lower the pH of the ensiling material as quickly as possible, while the presence of Clostridia contributes to a lower quality of the silage. Therefore, both genera were of interest in this study. Accordingly, the colonies grown on LAMVAB and SCHA agar were subjected to species identification using Matrix-Assisted Laser Desorption Ionisation–Time of Flight Mass Spectrometry (MALDI-TOF MS). The aim was to determine which species of the two genera were predominantly found in the samples examined (manure, grass and grass silage). For this purpose, colonies with different morphologies, obtained from manure, grass and grass silage, were extracted using the formic acid extraction method as described in the instruction manual (Bruker Daltonik GmbH, Germany). Samples were spotted in triplicate on a polished steel plate. The generated protein spectra were compared with the MALDI-TOF biotyper database (Bruker Daltonik GmbH, Germany).

2.6 | Statistical Analysis

Multiple linear regression models were used to analyse the effects of the three manure application methods tested on the number ($\log_{10}$ cfu/g) of bacteria (APC, LAB, *Lactobacillus* spp., *Enterobacteriaceae*, *E. coli*, yeasts and *Clostridium* spp.), while controlling for covariates such as harvests and year. Analysis of variance followed by post hoc tests (R Statistical Software, version 4.2.2, R Foundation for Statistical Computing, Vienna, Austria) was performed to determine statistical differences in microbial counts in grass and grass silage samples obtained with different manure application methods. Tukey's HSD correction was used as a post hoc test for multiple comparisons in relation to different combinations of application methods. The difference was considered statistically significant if the adjusted p value was below 0.05.

In addition, the Pearson's correlation coefficient (r, Microsoft Excel) was used to determine the correlation between counts of microorganisms found in the grass and in grass silage samples and between the microorganisms found in the grass silage samples. The strength of the correlation was interpreted as follows: $r = 0–0.19$ is considered very weak, 0.20–0.39 weak, 0.40–0.59 moderate, 0.60–0.79 strong and 0.8–1.0 very strong (Evans 1996). Subsequently, the p value was calculated based on a two-tailed t-test to determine whether the correlation was statistically significant ($p < 0.05$) (Dorn-In et al. 2023).

The statistical analysis regarding the difference in microbial counts in soil samples from each block was not carried out, as the number of soil samples ($n = 4$) per block was too small for this purpose and the samples were collected only at a single time point.

3 | Results and Discussion

3.1 | Microbiological and Molecular Examination

3.1.1 | Soil Samples

Table 2 shows the microbial count of soil samples collected in March 2020 prior to the beginning of the trial. The observations showed that the soils of all plots in the experimental field did not show any major differences in terms of the number of microorganisms; thus, there was no bias between plots regarding the levels of initial microbiota. In general, soil can be an important source of microbial contamination of grass, especially with *Enterobacteriaceae* and Clostridia (Östling and Lindgren 1991). The latter bacteria are widely distributed in soil (Palmer et al. 2019), where they play an important role in the decomposition of organic compounds (Hippe et al. 1992).

The microbiological quality of soils can be influenced by many factors, such as soil type, geographical region and manure application. As shown by Rammer et al. (1997), LAB counts in

TABLE 2 | Microbial count (means and standard deviation) of investigated samples and fertilised field plots.

Sample	Field	*n*	Culture (log_{10} cfu/g)						qPCR (log_{10} cfu/g)
Types	**Plot**	**Samples**	**APC**	**LAB**	***Lactobacillus***	***Enterobacteriaceae***	***E. coli***	**yeasts**	***Clostridium* spp.**
Soil*	Mineral	4	6.5 (±0.2)	2.9 (±0.2)	n.d.	3.2 (±1.5)	n.d.	3.3 (±1.5)	4.6 (±0.1)
	Broadcast	4	6.5 (±0.2)	3.3 (±0.2)	n.d.	2.5 (±1.8)	n.d.	3.1 (±1.4)	4.6 (±0.1)
	Trailing shoe	4	6.3 (±0.2)	3.0 (±0.6)	n.d.	4.5 (±0.2)	n.d.	2.4 (±1.0)	4.4 (±0.2)
	Disc injector	4	6.4 (±0.3)	3.0 (±0.4)	n.d.	4.2 (±0.4)	n.d.	2.1 (±0.7)	4.1 (±0.4)
Manure (M1–M5)		5	5.6 (±0.2)	3.6 (±0.8)	3.5 (±0.3)	4.9 (±2.3)	n.d.	≤ 2.3	5.4 (±0.3)
Grass (G0)*	Mineral	4	7.7 (±0.2)	3.2 (±1.2)	n.d.	3.0 (±1.4)	n.d.	5.8 (±0.1)	2.6 (±0.1)
	Broadcast	4	7.5 (±0.3)	1.5 (±0.6)	n.d.	3.3 (±0.7)	n.d.	5.7 (±0.1)	2.4 (±0.2)
	Trailing shoe	4	8.2 (±01)	2.3 (±0.9)	n.d.	3.3 (±0.6)	n.d.	5.5 (±0.2)	2.6 (±0.2)
	Disc injector	4	7.8 (±0.1)	2.3 (±0.9)	n.d.	3.6 (±0.6)	n.d.	5.7 (±0.1)	2.6 (±0.2)
Grass (G1–G5)	Mineral	20	8.0 (±0.7)	4.2 (±1.4)	1.7 (±1.3)	5.4 (±1.3)^{a}	1.5 (±1.1)	5.7 (±0.3)	1.7 (±0.9)^{a}
	Broadcast	20	7.8 (±0.6)	3.4 (±1.4)	1.5 (±1.2)	4.9 (±1.4)^{b}	1.4 (±0.9)	5.3 (±0.9)	3.3 (±1.2)^{b}
	Trailing shoe	20	7.9 (±0.7)	4.0 (±1.5)	1.8 (±1.5)	5.3 (±1.4)	1.7 (±1.3)	5.3 (±1.5)	3.2 (±1.0)^{b}
	Disc injector	20	7.9 (±0.9)	4.0 (±1.5)	2.0 (±1.7)	5.1 (±1.7)	1.8 (±1.4)	5.1 (±1.5)	2.6 (±1.0)^{c}
Grass silage (S1–S5)	Mineral	20	5.8 (±1.0)	6.8 (±0.7)	5.9 (±1.8)	n.d.	n.d.	1.2 (±1.0)	3.3 (±1.0)^{a}
	Broadcast	20	5.7 (±0.7)	6.4 (±0.7)	5.7 (±1.4)	n.d.	n.d.	n.d.	3.7 (±0.8)
	Trailing shoe	20	5.9 (±0.6)	6.6 (±0.9)	6.3 (±1.1)	n.d.	n.d.	n.d.	3.9 (±0.9)^{b}
	Disc injector	20	6.1 (±0.7)	6.7 (±0.8)	6.3 (±1.1)	n.d.	n.d.	n.d.	3.7 (±0.5)

Note: n.d., not detected (< 2.0 log_{10} cfu/g); for calculation of average and standard deviation, the value of 1.0 log_{10} cfu/g was used for these samples. ^{a,b,c}Different superscripts within a microbial group and a sample type indicate that the values are statistically significantly different ($p < 0.05$).

Abbreviations: APC, aerobic plate count; LAB, lactic acid bacteria.

*Samples (soil and grass G0) were collected directly before the experiment started, thus before the manure (M1) was distributed for the first time.

soil samples increased from 2.8 to 3.4 and 4.3 $\log_{10}$ cfu/g in the first, second and third experimental years, respectively, after 3 years of two manure applications each. In that study, the counts of Clostridia (3.9, 4.0 and 3.6 $\log_{10}$ cfu/g, respectively) and *Enterobacteriaceae* (5.0, 4.8 and 5.0 $\log_{10}$ cfu/g, respectively) were quite similar in the three experimental years (Rammer et al. 1997). In comparison, in the present study, the counts were 6.4 (±0.2) $\log_{10}$ cfu/g for APC, 3.0 (±0.4) $\log_{10}$ cfu/g for LAB and 4.4 (±0.3) $\log_{10}$ cfu/g for Clostridia (see Table 2). *Enterobacteriaceae* counts varied between plots and ranged from not detected (< 2.0) to 4.7 (3.6 ± 1.4) $\log_{10}$ cfu/g. This observation was also made for yeasts, that ranged from not detected (< 2.0) to 4.0 (2.7 ± 1.2) $\log_{10}$ cfu/g. *Lactobacillus* spp. (determined on LAMVAB agar) and *E. coli* (determined on Rapid *E. coli* agar) were not detected in soil samples in this study (see Table 2). A very low number of *E. coli* in soils that have not been fertilised with manure for a long time was also observed by Ingham et al. (2004). The authors concluded that *E. coli* in the soil decreased by up to 3.0 $\log_{10}$ cfu/g from an initial value of 4.2–4.4 $\log_{10}$ cfu/g after 90 days of fertilisation with fresh manure. After 100 days, they were only detected in enriched soil samples. Similar results were observed by Sinton et al. (2007) when fresh cow faeces were scattered on the field and the cow faeces were collected until they had disintegrated and/or could no longer be distinguished from the underlying soil, which took place over a period of 5–6 months. The authors found that intestinal bacteria such as *E. coli*, faecal streptococci and pathogens such as *Salmonella* spp. and *Campylobacter jejuni* decreased significantly in the residues of cow faeces (Sinton et al. 2007).

The soil samples in the present study were taken only once in March 2020, before the first manure application (M1). The last manure fertilisation of the experimental field before the trial took place in autumn 2019, which is more than 100 days. Therefore, the chance of detecting *E. coli* and probably some other bacteria in soils originally derived from manure is very low. The death or reduction of manure-borne pathogens occurs once the manure is incorporated into the soil, where environmental conditions are likely to be less than optimal for them, such as temperature, weather conditions or the presence of other microorganisms (Ingham et al. 2004; Sinton et al. 2007).

3.1.2 | Manure Samples (M1–M5)

In general, composting or, alternatively, storing manure for a long period can reduce the number of undesirable microorganisms in manure or slurry (Heinonen-Tanski et al. 2006; Ingham et al. 2004; Wang et al. 2004). These manure treatment methods should be practiced to reduce cross-contamination of pathogens from manure to vegetables or, as in this study, to grassland (Ingham et al. 2004). In the present study, the manure was applied to experimental fields after being stored in a covered manure pit for approximately 5 months (M1) and 17 months (M5). The manure samples were taken before every spreading on the plots. The bacterial counts were 5.6 (±0.2) $\log_{10}$ cfu/g for APC, 3.6 (±0.8) for LAB, 3.5 (±0.3) for *Lactobacillus* and 4.9 (±2.3) $\log_{10}$ cfu/g for *Enterobacteriaceae* (see Table 2). *E. coli* was not detected and yeasts were only found in one manure sample (M5, 2.3 $\log_{10}$ cfu/g).

Since the manure in this study was stored for a long period of time before it was used, it contained relatively low levels of microorganisms. In fresh cow faeces, the number of LAB and Enterobacteriaceae was 6.1 (5.0–6.7) and 6.2 (4.8–7.0) $\log_{10}$ cfu/g, respectively (Östling and Lindgren 1991), the number of E. coli and coliforms was 6.8 and 7.1 $\log_{10}$ cfu/g, respectively (Wang et al. 2004), the total bacterial count was up to 7.6 $\log_{10}$ cfu/g (Hongal et al. 2023) or between 8.0 and 10.0 $\log_{10}$ cfu/g (Heinonen-Tanski et al. 2006). However, at 5.4 (±0.3) $\log_{10}$ cfu/g as determined by the qPCR (see Table 2), the clostridial count in the manure in this study was higher than that of Östling and Lindgren (1991), in which clostridial spores were found between 2.3 and 4.7 $\log_{10}$ cfu/g (average 4.1 $\log_{10}$ cfu/g) as determined by culture methods. Manure can be a source of LAB and *Lactobacillus* spp. for grass, which are needed to lower the pH value of silage by producing lactic acid. However, high concentrations of *Enterobacteriaceae* and Clostridia in manure usually have a negative impact on the hygiene and quality of grass and grass silage (Östling and Lindgren 1991).

As described in the section on soil samples, Clostridia are commonly found in soil. Their presence in manure results from animals ingesting grass or silage contaminated with soil. In turn, applying manure with a high Clostridia load to fertilise grassland leads to an increase in the number of Clostridia in soil. However, this depended on the hygiene and the manure management practices of the farms, which resulted in varying Clostridia counts in manure among farms. In the present study, the clostridial count in manure was approximately 1.0 $\log_{10}$ cfu/g higher than in soil samples (see Table 2). Since there is a lack of data with comparable samples and quantification methods, it remains uncertain whether the clostridial load in the manure sample used for fertilising grassland in the present study is high or low. It was also found that the manure contained not only a higher number of pathogenic or spoilage bacteria (*Enterobacteriaceae* and Clostridia) compared to the soil samples but also the necessary bacteria for the ensiling process (LAB, *Lactobacillus*; see Table 2).

3.1.3 | Grass Samples (G0–G5)

The summary of the microbial count in wilted–chopped grass samples is demonstrated in Table 2. The count of these microorganisms at the individual sampling times is shown in Figure 2 and Table S1.

3.1.4 | Grass G0

Similar to the soil samples, grass G0 from all plots collected in March 2020 (before the first manure application) showed no significant differences in the counts of the investigated microorganisms. The microbial load was on average 7.8 (7.2–8.3) $\log_{10}$ cfu/g for APC, 2.3 (< 2.0–4.4) $\log_{10}$ cfu/g for LAB, 3.3 (< 2.0–4.5) $\log_{10}$ cfu/g for *Enterobacteriaceae*, 5.7 (5.2–6.0) $\log_{10}$ cfu/g for yeasts and 2.5 (2.1–2.9) $\log_{10}$ cfu/g for Clostridia (see Table 2). In general, the microbial load on fresh grass samples is influenced by harvest time, manure application and weather conditions; therefore, data between studies is expected to vary over a wide range and is not well comparable. For instance, Pahlow

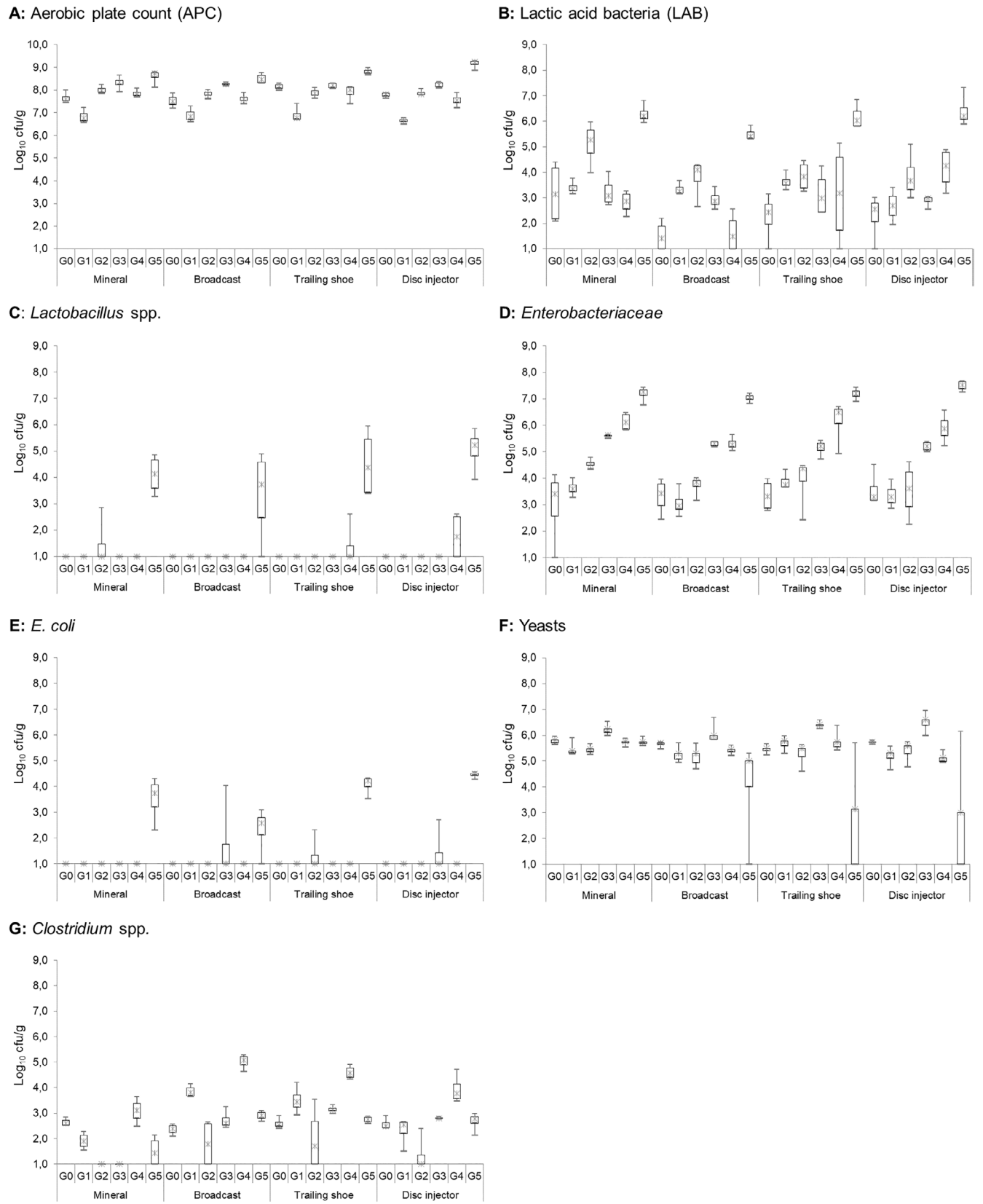

FIGURE 2 | Microbial count of grass sample G0–G5 from the mineral fertiliser control group and three manure application methods (for more information, see Table S2).

et al. (2003) found > 9.0 $\log_{10}$ cfu/g for APC, 1.0–6.0 $\log_{10}$ cfu/g for LAB, 3.0–6.0 $\log_{10}$ cfu/g for *Enterobacteriaceae* and 2.0–3.0 $\log_{10}$ cfu/g for clostridial spores.

The number of yeasts in the grass in this study (5.1–5.8 $\log_{10}$ cfu/g, see Table 2) was higher than that reported by Pahlow et al. (2003) (3.0–5.0 $\log_{10}$ cfu/g) but in the same range as that found by

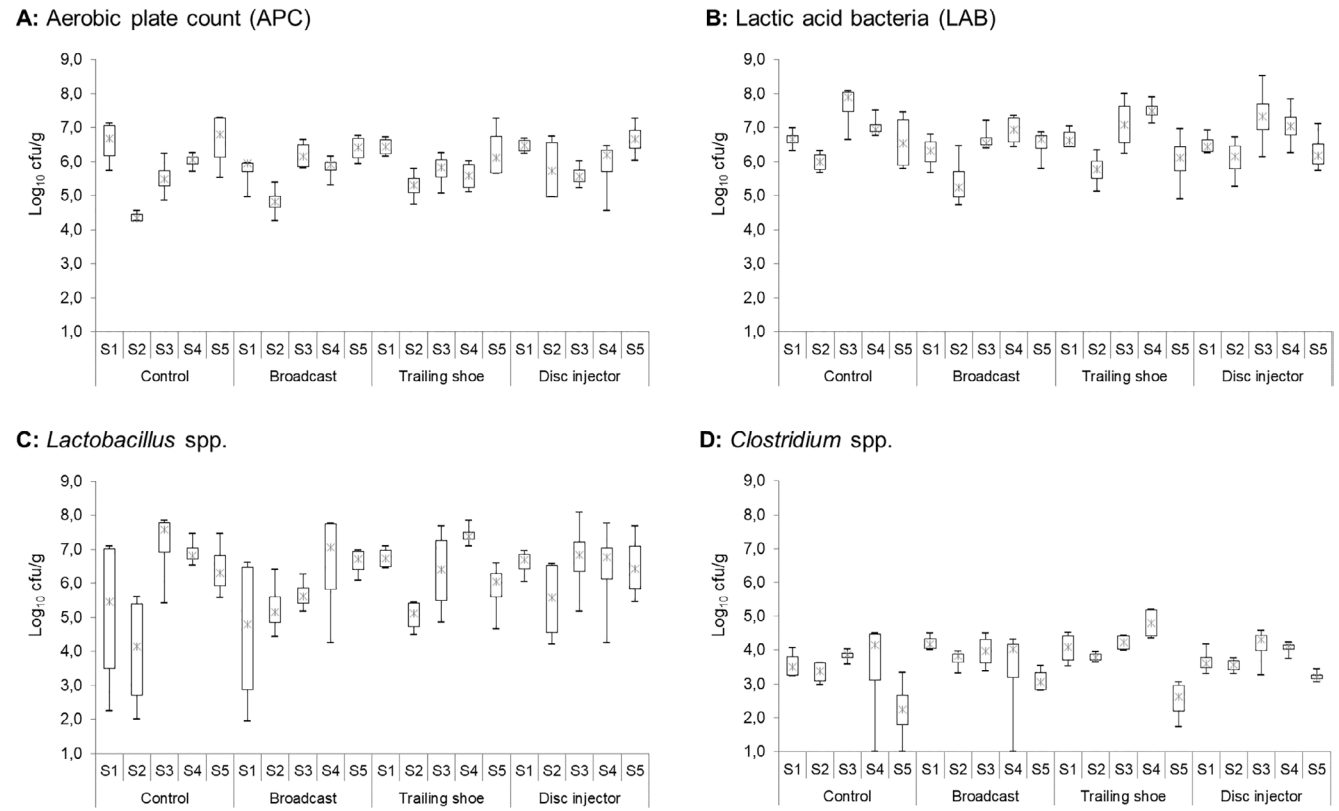

FIGURE 3 | Microbial count of grass silage samples S1–S5 from the mineral fertiliser (control group) and three manure application methods: *Enterobacteriaceae*, *E. coli*, and yeasts were not found or detected at a very low level (see Tables 2 and S2).

Persson (2015) (4.6–5.9 $\log_{10}$ cfu/g). Another study reported that, on standing crops, the number of *Enterobacteriaceae* was 4.5–5.5 $\log_{10}$ cfu/g, clostridial spores were found at <1.7 $\log_{10}$ cfu/g (Li et al. 2020; Östling and Lindgren 1991) and LAB levels were at 2.6–3.1 $\log_{10}$ cfu/g (Östling and Lindgren 1991), <2.0–5.0 $\log_{10}$ cfu/g (Carvalho et al. 2021) or 4.7 ± 0.4 $\log_{10}$ cfu/g (Li et al. 2020). LAB and *Lactobacillus* spp. are usually considered desirable microorganisms in soil and in grass as they can contribute to the protection of grass against plant diseases (Jaffar et al. 2023). According to Pahlow et al. (2003), LAB counts are usually very low or are not detectable during the cold period and only start to be detectable when the average temperature exceeds 10°C. In addition, many LAB species on the plant surface may be in a viable but non-culturable (VBNC) state, leading to an underestimation of LAB numbers on plants or grass (Pahlow et al. 2003).

3.1.5 | Influence of Harvest Times on the Microbial Count of Grass G1–G5

In addition to manure and soil, other factors such as year, season, temperature and weather conditions can also influence the bacterial counts in the grass. During the trial period (see Section 2.2), there was little rainfall in spring and summer 2020 (which is normal for this region), while 2021 was characterised by warm temperatures, high humidity and numerous rainfalls. These conditions can influence the number of microorganisms in the grass, especially APC, LAB and Clostridia, as their number varies between harvest times. This was observed in all manure application variations, including the control group (see Figure 3). APC and LAB have a similar tendency to increase or decrease at each harvest time and reached their highest values at the last harvest (grass G5, Figure 2A,B; Table S2). Although the number of Clostridia in the manure samples was relatively high (5.4 ± 0.3 $\log_{10}$ cfu/g), they were found in grass samples at a low level (from not detected up to 4.2 $\log_{10}$ cfu/g), except in grass G4, where the number of Clostridia was higher than in the other harvests (up to 5.3 $\log_{10}$ cfu/g, Figure 2G; Table S2).

As shown in Figure 3, yeast counts seemed not to be affected by the weather conditions and manure application since grass G1–G4 from all fertilising groups showed similar counts to grass G0. However, no yeasts were cultured from some samples of grass G5. While the numbers of APC, LAB and Clostridia varied during the experimental periods, *Enterobacteriaceae* were the only microbial group that continuously increased from approximately 3.0 $\log_{10}$ cfu/g (grass G0) to 7.0 $\log_{10}$ cfu/g in G5 for all manure application variations, including the control group. Probably due to the high humidity in July 2021, *Lactobacillus* spp. and *E. coli* could be isolated from all G5 samples but only from a few samples from the other harvests (G0–G4, see Figure 2C,E and Table S2).

As the number of APC, LAB, *Enterobacteriaceae* and Clostridia in grass samples is dynamic, their correlation was analysed. While APC, LAB and *Enterobacteriaceae* have a moderate to strong positive correlation ($r = 0.52$–0.76, $p < 0.05$) regarding their counts in the grass samples, a weak negative correlation was only found between LAB and Clostridia ($r = -0.311$, $p < 0.05$). This indicates that conditions that have a positive effect on the growth of LAB in grass may not apply to Clostridia.

TABLE 3 | Correlation (r) between counts of microorganisms in grass and in grass silage.

		Grass silage							
		APC		LAB		Lactobacillus		Clostridium	
		r	p	r	p	r	p	r	p
Grass	APC	0.030	0.794	0.038	0.739	0.144	0.202	−0.301	0.007
	LAB			−0.228	0.042	−0.069	0.543	−0.417	< 0.001
	Lactobacillus					0.100	0.376	−0.518	< 0.001
	Clostridium							0.281	0.011
Grass silage	APC			0.280	0.012	0.384	< 0.001	−0.193	0.087
	LAB					0.671	< 0.001	0.260	0.020
	Lactobacillus							0.099	0.383

Abbreviations: APC, aerobic plate count; LAB, lactic acid bacteria.

3.1.6 | Influence of Manure Application Methods on the Microbial Count of Grass G1–G5

Regardless of the weather conditions and the year analysed, the grass samples (G1–G5) from the control group contained *Enterobacteriaceae* at a statistically higher level than the broadcast method, although the log difference was quite small (5.4 vs. 4.9 $\log_{10}$ cfu/g, Table 2). The number of Clostridia in the grass samples from the control group (1.7 $\log_{10}$ cfu/g) was statistically lower than in all three other manure application methods ($p < 0.05$, Table 2).

When comparing the three manure application methods, the only significant difference between application methods was in the Clostridia numbers. The disc injector method resulted in statistically significantly fewer Clostridia than the broadcast and trailing shoe methods (2.6 vs. 3.3 and 3.2 $\log_{10}$ cfu/g, $p < 0.05$, Table 2). While these differences are statistically significant, the numerical difference remains rather low at 0.7 $\log_{10}$ cfu/g. The interpretation of any biological relevance of these differences should therefore be evaluated with care. These results suggest that manure application to grassland may increase the number of Clostridia in wilted-chopped grass samples but not with the other investigated microbial groups.

3.1.7 | Silages S1–S5

The summary of microbial counts in all grass silage samples is demonstrated in Table 2. The details of the microbial loads in the grass silage samples at each individual time point are shown in Figure 3 and Table S2.

Overall, APC, LAB, *Lactobacillus* spp. and *Clostridium* spp. were found in almost all grass silage samples, while *Enterobacteriaceae*, *E. coli* and yeasts were either not present or only in very small quantities in individual samples (see Table 2 and Figure 3). This indicates that the condition of ensiling was favourable for LAB and that the growth of *Enterobacteriaceae* and yeasts was inhibited by the decreasing pH value due to the lactic acid production of LAB. Both microbial groups (*Enterobacteriaceae* and yeasts) are known to cause aerobic deterioration of the silages (Pahlow et al. 2003).

3.1.8 | Correlation Between the Bacterial Loads in the Grass and in the Grass Silage Samples

The bacterial loads detected in grass silage samples were relatively different between harvest times but quite similar between grass silage samples harvested at the same time for all three manure fertilisation variations and the control (see Table 2). APC (5.7–6.1 $\log_{10}$ cfu/g) was found on average two log levels lower than in grass samples; LAB (6.4–6.8 $\log_{10}$ cfu/g) and *Lactobacillus* (5.7–6.3 $\log_{10}$ cfu/g) were on average two and three log levels higher than in the grass samples. In contrast, Clostridia (3.3–3.9 $\log_{10}$ cfu/g) were slightly higher than those found in the grass samples (see Table 2).

LAB and *Lactobacillus* spp. are important for the ensiling process to lower the pH value of the grass silage to a level that inhibits the growth of other undesirable bacteria, including Clostridia. As shown in Table 3, a high number of LAB and *Lactobacillus* spp. in grass leads to a significantly lower clostridial count in grass silage samples ($r = -0.42$ and -0.52; $p = 1.2 \times 10^{-4}$ and 8.5×10^{-7}, respectively). On the other hand, grass with a high clostridial number can also lead to a high Clostridia load in the grass silage ($r = 0.28$, $p = 0.01$). This was in contradiction to the results of the correlation analysis of the bacteria found in the grass silage samples, as grass silages with high LAB counts (but not *Lactobacillus* spp.) have a statistically significant tendency to contain high Clostridia loads ($r = 0.26$, $p = 0.02$). This indicates that the conditions that favour the growth of LAB in silage may also apply to some *Clostridium* species, although not all.

3.1.9 | Correlation Between the Bacterial Loads in Grass Silage Samples From Different Manure Application Methods

No statistically significant difference was found in any bacterial loads when comparing the different manure application methods. A statistically significant difference ($p < 0.05$) regarding the clostridial number was only found between the mineral and the trailing shoe fertilised samples (3.3 vs. 3.9 $\log_{10}$ cfu/g, see Table 3). As with the differences in Clostridia numbers between grass samples, the numerical difference of 0.6 $\log_{10}$ cfu/g

is small and also well within the standard deviation of the samples. Therefore, its biological significance should be interpreted with caution.

In similar studies conducted in other countries in Europe, for example, in the United Kingdom, the authors concluded that grass silage from trailing shoe fertilisation achieved the best quality in terms of chemical constituents compared to surface broadcast application and injection fertilisation (Laws et al. 2002). However, the microbiological quality of the grass silage was not analysed in that study. In addition, Laws et al. (2002) concluded that the injection-based methods can damage the taller swards through the injection tines, leading to contamination of the forage with soil and manure and consequently poor silage fermentation. Using the broadcast method, the quality of silage from plots where manure was applied 2 weeks before harvest showed characteristics indicative of clostridial growth, with a high pH value and low lactic acid content (Laws et al. 2002). Similarly, in a study conducted in Switzerland, higher clostridial counts were found in grass from broadcast and band-spread compared to grass from trailing-shoe fertilisation when the manure was applied late after cutting (grass about 10 cm high) (Wyss et al. 2017). In addition, the authors found that the correlation between the number of clostridial spores (determined using the most probable number method) in the grass and the butyric acid content in the silage was very low (Wyss et al. 2017). However, the mentioned study investigated the Clostridia load only in the grass but not in the silage samples.

Another study carried out in Austria compared nitrogen losses and the microbial count of grass between three broadcasting (head-, low pressure- and pendulum distributors) and three near-ground distribution techniques (band spreading-, trailing shoe- and disc injection) (Pöllinger et al. 2018). The authors concluded that broadcasting-based techniques lead to higher nitrogen losses and higher APC counts on grass (average 6.0 $\log_{10}$ cfu/g) than the near-ground distribution techniques (average 5.0 $\log_{10}$ cfu/g). However, there was no difference between the application techniques with regard to *Enterobacteriaceae* and clostridial counts (approximately 3.0–4.0 and 4.0–5.0 $\log_{10}$ cfu/g, respectively) (Pöllinger et al. 2018).

3.1.10 | Explanatory Note

According to the different fertilising times after grass cutting, the weather condition, the spreading technique and the different circumstances during fertilisation, as mentioned in the previous paragraphs, our results should be carefully interpreted when comparing with other studies. In the present study, the manure (M1–M3, see Table 1) was applied on the same day the grass was cut or 5 months (M4, due to wintertime, November to March) and 3 days (M5) after the grass was cut. In general, grass silage quality is influenced by many factors, including the microbial community and metabolites present during the ensiling process (Li et al. 2020), the composition of the plant material and the application of appropriate silage-making management, such as the speed of packing, pack density, type of additive used, chop length, covering management and silo management during feed-out, which can also affect grass silage fermentation and its subsequent quality (Driehuis et al. 2018; Kung et al., Kung Jr. et al. 2018). Based on the correlation analysis in this study, high levels of LAB and *Lactobacillus* spp. in grass samples contributed to the inhibition of clostridial growth regardless of the manure application methods. Therefore, the use of a suitable ensiling agent containing LAB and *Lactobacillus* spp., as observed in other studies, represents a good option to ensure good silage quality. In addition to the presence of pathogens and spoilage microorganisms, another important point is the quality of the silage in terms of chemical composition. The chemical analysis data were not available in this study, as the focus of this project was on comparing the microbial quality of grass and grass silage samples obtained using different manure application methods.

3.2 | MALDI-TOF and Species Identification

Using MALDI-TOF MS, 168 colonies on LAMVAB agar and 119 colonies on SCHA agar were identified down to the species level in the genera *Lactobacillus* spp. and *Clostridium* spp., respectively. Table 4 shows the summary of identified species from three sample types, namely manure, grass and grass silage.

The homofermentative (e.g., *L. acidophilus, L. salivarius* and *L. lactis*) and facultative heterofermentative LAB (e.g., *L. plantarum, L. rhamnosus, L. paracasei, L. casei, L. farraginis* and *L. parafarraginis*) are generally used in the ensiling process to lower the pH value by producing lactic acid (Carvalho et al. 2021; Muck et al. 2018). Lowering the pH value is a crucial step in the fermentation process to inhibit the growth of *Enterobacteriaceae*, Clostridia and other undesirable microorganisms, leading to a reduction in proteolysis and dry matter losses (Carvalho et al. 2021; Muck et al. 2018). In this study, the most abundant *Lactobacillus* spp. found in manure samples were *L. reuteri* and *L. buchneri*, followed by *L. paracasei*, while in grass samples *L. curvatus*, *L. plantarum* and *L. sakei* were the dominant species. In grass silage samples, *L. paracasei* dominated by far, followed by *L. acidipiscis*, *L. buchneri* and *L. plantarum*. The species *L. acidipiscis* could only be isolated from grass silage samples; otherwise, almost all *Lactobacillus* spp. found in manure or grass samples were also found in grass silage samples.

A high diversity of *Clostridium* species was found in the manure samples. Out of 13 clostridial colonies recovered from manure, 3 (23.1%) were identified as *C. perfringens*. This species is a potential pathogen causing feed poisoning, as it can produce more than 20 virulent toxins (Goldsztejn et al. 2020). In the grass samples, by far the most abundant species was *C. isatidis*. There are not many reports about this *Clostridium* species, but it is known as an indigo-reducing moderate thermophile *Clostridium* (Padden et al. 2000).

In this study, the *Clostridium* spp. found in manure samples were not found in grass samples and species found in grass samples were not detected in grass silage samples. Out of 16 identified species, only two species (*C. tyrobutyricum* and *C. butyricum*) were found in two different sample types, namely in manure and grass silage samples. The species *C. tyrobutyricum* was predominantly found in grass silage samples, followed by *C. sporogenes* and *C. butyricum*, which is in line with the results of some other studies (Driehuis et al. 2016; Li et al. 2020). These three *Clostridium* species are non-pathogenic but are considered

TABLE 4 | Species belonging to *Lactobacillus* spp. ($n = 168$) and *Clostridium* spp. ($n = 119$) identified using MALDI-TOF MS.

Genus	Identified species	Sources			*n* total
		Manure	Grass	Grass silage	
Lactobacillus spp.	*L. acidipiscis*			36	36 (21.4%)
	L. brevis	1	1	3	5 (3.0%)
	L. buchneri	9		16	25 (14.9%)
	L. coryniformis	1	2	2	5 (3.0%)
	L. curvatus	1	5		6 (3.6%)
	L. farciminis			1	1 (0.6%)
	L. hilgardii	1			1 (0.6%)
	L. paracasei	6	1	47	54 (32.1%)
	L. plantarum	1	4	13	18 (10.7%)
	L. reuteri	9		4	13 (7.7%)
	L. sakei		4		4 (2.4%)
Clostridium spp.	*C. beijerinckii*	1			1 (0.8%)
	C. butyricum	2		9	11 (9.2%)
	C. celerecrescens			1	1 (0.8%)
	C. cellobioparum		1		1 (0.8%)
	C. cochlearium	2			2 (1.7%)
	C. drakei			1	1 (0.8%)
	C. indolis	1			1 (0.8%)
	C. isatidis		10		10 (8.4%)
	C. jejuense	1			1 (0.8%)
	C. perfringens	3			3 (2.5%)
	C. sardiniense		1		1 (0.8%)
	C. sartagoforme	1			1 (0.8%)
	C. sporogenes			15	15 (12.6%)
	C. sporosphaeroides	1			1 (0.8%)
	C. subterminale			1	1 (0.8%)
	C. tyrobutyricum	1		67	68 (57.1%)

spoilage agents for food and feed. The species *C. tyrobutyricum* and *C. butyricum* can tolerate low pH values down to 4.2 and 4.5, respectively (König et al. 2017), which may explain their over-representation in grass silage samples. Especially *C. tyrobutyricum* is considered an important bacterium causing anaerobic spoilage of food and feed due to its ability to produce butyric acid, acetic acid, amines, H_2, NH_3 and CO_2 (Driehuis and Elferink 2000). This species can cross-contaminate from feed (silage) to milk, which later plays a role as a causative agent of late blowing in hard and semi-hard cheeses (Esteban et al. 2020; Klijn et al. 1995), resulting in considerable losses for the dairy industry. The species *C. sporogenes* can grow in grass silage when the pH value is higher than 5.0. This species ferments both carbohydrates and proteins resulting in undesirable products similar to those produced by *C. tyrobutyricum* (Driehuis et al. 2018). For this reason, some countries, such as Switzerland, encourage and subsidise farmers to not feed cows, sheep or goats with silage when their milk is processed into cheese (MSV 2023).

4 | Conclusion

The microbiological quality of grass and grass silage obtained from three manure application methods (broadcast with splash plate, trailing shoe and disc injector) and a control group with mineral fertiliser (CAN) was compared. For this purpose, the numbers of seven microbial groups were analysed by culture (APC, LAB, *Lactobacillus* spp., *Enterobacteriaceae*, *E. coli* and yeasts) and qPCR (*Clostridium* spp.). When comparing the three manure application methods, only the number of *Clostridium*

spp. in wilted-chopped grass obtained from the disc injector was statistically significantly lower than the splash plate and trailing shoe methods ($p < 0.05$). No statistically significant difference between the three manure application methods was observed regarding the counts of the examined microorganisms in grass silage. These results show that the trailing shoe and disc injector methods, when applied in dry areas such as Lower Franconia (Bavaria, Germany), do not increase the number of examined microorganisms on the grass and in the grass silage compared to the broadcast (splash plate) method.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Grass samples G0–G5 obtained from each plot and their microbiological quality. **Table S2:** Grass silage samples S1–S5 obtained from each plot and their microbiological quality.