Fermentation Quality and Bacterial Ecology of Grass Silage Modulated by Additive Treatments, Extent of Compaction and Soil Contamination

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Fermentation Quality and Bacterial Ecology of Grass Silage Modulated by Additive Treatments, Extent of Compaction and Soil Contamination

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Abstract: New technologies related to the identification of bacterial communities in fresh forage and silage may give valuable detailed information on the best practices to produce animal feeds. The objective was to evaluate how management conditions during silage making manipulate the profile of bacterial communities and fermentation quality of grass silages. Silages were prepared from mixed timothy and meadow fescue grass using two compaction levels. As an additional treatment the grass was contaminated with soil and feces prior to tight compaction. Four additive treatments with different modes of action were applied: control without additive, formic acid-based additive, homofermentative lactic acid bacteria and salt-based additive. After 93 days the silos were opened, samples were taken and routinely analyzed. DNA extraction was carried out and PCR amplification of the bacterial 16S rRNA gene V4 region was performed using universal primers. The silage pH was higher for loose than tight compaction and higher for non-contaminated than for contaminated silages. Great shift was observed in bacterial profiles from fresh material towards silage. *Lactobacillus* genus was barely found on the relative abundance of fresh grass but became predominant in the final silage along with *Sphingomonas* genus. Use of additives improved fermentation quality and modified the bacterial profiles of grass ensiled under different management conditions.

Keywords: *Festuca pratensis*; *Lactobacillus* genus; microbial ecology; microbiome; metagenome; *Phleum pratense*; silage additive

1. Introduction

Fresh plant material contains a large variety of epiphytic microorganisms such as bacteria, yeasts and molds and silage fermentation is a result of the activity of bacterial communities [1]. Identifying the bacterial community diversity of fresh plant material and silages treated with different additives and/or untreated provides new insight into understanding the process of silage fermentation [2–4]. Each specific bacterial community can shift the nutritional composition and hygienic quality of the feed and influence ensiling losses. Optimally, silage fermentation is dominated by *Lactobacillus* spp., but there are numerous different bacteria present, some of which are detrimental causing spoilage and fermentation losses.

Until recently it was difficult and expensive to identify the entire bacterial community in silage, but sequencing techniques are more efficient and cost-effective than previously [5]. These technologies related to whole comparative genomics, metagenomics and metatranscriptomics can provide us more detailed information about microbiota of harvested forage

and silage than classic microbiology [2]. This information could help us to characterize the role of silage additives and epiphytic bacteria in the ensiling process and improve silage quality as well as decrease the abundance of yeasts, molds and unwanted bacteria in silage.

General good management practices in ensiling of forages include tight compaction to ensure anaerobic conditions and avoiding contamination to prevent inoculation with spoilage microbes. Additives are commonly used to improve the fermentation quality of the forage [6]. Information about the microbiome and metabolome of silages could be used to obtain better fermentation quality, to make functional silages to increase animal health and welfare and ultimately even improve milk quality for humans. However, currently there are no such practical applications available. There is still only limited information available in literature about the complexity of bacterial communities of grass silages and how silage management factors can produce microbiomes that are more favorable to high-quality silage production. Thus, the objective of this research was to evaluate how different types of silage additives can manipulate the ensiling process and, additionally, to trace the profile of bacterial communities of grass silages under varying management conditions represented by two levels of compaction and contamination. Preliminary results of the current experiment have been published by Franco et al. [7,8].

2. Materials and Methods

2.1. Raw Material for Silage Making

Mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) grass was harvested on 4 June 2018 at the Natural Resources Institute Finland (Luke) in Jokioinen, Finland (60°48′ N, 23°29′ E) from a grass ley established five years earlier. Grass was cut with a mower conditioner (JF GMS 3200 Topflex, JF-Fabriken- J Freudendahl A/S, Sonderborg, Denmark and Krone EC 32 CV in front hitch, Maschinefabrik Bernard Krone GmbH, Spelle, Germany) and harvested after a short wilting period of 1 to 2 h. Grass was precision chopped using farm scale machinery (JF FCT 1350, JF-Fabriken- J Freudendahl A/S, Sonderborg, Denmark) and transported to the laboratory without any additive. Raw material samples were taken before treatment application in order to evaluate chemical composition, microbial quality and bacterial communities of grass before ensiling.

2.2. Treatments and Experimental Procedures

Silages were prepared using loose and tight compaction levels. In order to produce the loose silages, an 8 kg lead plummet used for grass compaction into cylindrical silos was dropped to the grass only twice after every handful of grass, while in the tight compaction the lead plummet was dropped 10 times after every handful of grass. To challenge the effect of additives on fermentation quality of a low-hygienic quality raw material, contamination with soil from a slurry-treated area was conducted for the tightly compacted silos. Soil was collected from a field two days after the area was treated with dairy cow slurry. Additional fresh cow feces were also added to the contaminant solution. This resulted in three different types of ensiling management: loose compaction, tight compaction and tight compaction with soil + feces contamination. For all management options, four additive treatments were applied including:

1. Control without additive (Control; tap water)
2. Formic acid (FA) based additive at 5 L/ton of fresh matter (formic acid, propionic acid, sodium formate, and potassium sorbate; AIV Ässä Na, Eastman, Oulu, Finland)
3. Homofermentative lactic acid bacteria (LAB) at 1 g/ton of fresh matter providing 10^5 cfu per g fresh grass (*Lactobacillus plantarum*, 1.0×10^{11} cfu/g; KOFASIL® LAC, Addcon, Bitterfeld-Wolfen, Germany)
4. Salt based additive (SALT) at 2 L/ton of fresh matter (sodium nitrite, sodium benzoate and potassium sorbate; Safesil Challenge, Salinity AB, Göteborg, Sweden)

The grass was carefully mixed and divided into 36 batches of 15 kg each to which additive treatments were carefully and evenly applied. All additives were diluted with tap water so that the amount of liquid applied was 17 L per ton. Grass was packed into

cylindrical pilot scale silos with 12-L capacity using three replicates per treatment. Silos were capped with a plastic cover, plastic lid, a weight and a water lock, and then stored at room temperature with protection from light and opened after an ensiling period of 93 days. The height and weight of silage were measured before opening the silos in order to calculate the density of the silage. Silos were also weighed immediately after filling for calculation of ensiling losses according to Knicky and Spörndly [9] by using weight loss of silage during ensiling as an estimate of CO_2 production. For each mole of CO_2 produced during ensiling, 1 mole of H_2O is also produced. Thus, each gram of weight decrease because of CO_2 losses means that 0.44 g of dry matter (DM) was lost as water. Total DM loss (g/kg DM) was then estimated to be the decrease in weight of the silo multiplied by 1.44. The top layer of the silos and visually spoiled silage were discarded, and the rest of silage was carefully mixed, and representative samples were taken.

2.3. Laboratory Analyses

The DM content of silages and the raw material were determined by drying samples at 105 °C for 16 h and corrected for volatile compounds [10]. Crude protein (CP) was determined according to AOAC [11] method 968.06 (correction factor 6.25 × N, using Leco FP 428 nitrogen analyzer [Leco, St. Joseph, MI, USA]), and ash by method 942.05. Water soluble carbohydrates (WSC) were determined according to Somogyi [12], volatile fatty acids according to Huhtanen et al. [13], lactic acid according to Haacker et al. [14] and ammonia according to McCullough [15]. In vitro organic matter digestibility was determined according to Nousiainen et al. [16] with a correction equation of pepsin-cellulase solubility to in vivo digestibility by using data from Finnish in vivo digestibility trials [17]. Neutral detergent fiber (NDF) was determined by using ANKOM 220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) according to Van Soest et al. [18] using sodium sulphite and expressed without ash. Ethanol was detected with a commercial kit (Cat. No. 10176290035, Roche Diagnostics, Basel, Switzerland) by using a spectrophotometer according to manufacturer instructions. Buffering capacity (BC) of raw material was analyzed according to Weissbach et al. [19]. Fermentation coefficient (FC) was calculated based on DM, WSC and BC of raw material using following formula: FC = DM + (8 × WSC/BC) [20]. Quantitative estimates of the effects of silage characteristics on silage intake were expressed as silage DM intake (SDMI) index, which was calculated according to Huhtanen et al. [21].

Yeasts and molds were enumerated by cultivation on Dichloran Rose Bengal Chloramphenicol Agar (Lab217, Lab M Ltd., Lancashire, UK) with 50 µg/mL of oxytetracycline hydrochloride (AppliChem BioChemica A5257). The plates were incubated at 25 °C and visually counted after three and five days. Zearalenone and deoxynivalenol mycotoxins were determined using commercial kits RIDASCREEN®FAST Zearalenon and RIDASCREEN®FAST DON (R-Biopharm AG, Darmstadt, Germany), respectively. Both kits evaluate the presence of mycotoxins through a competitive enzyme immunoassay for the quantitative analysis of zearalenone and deoxynivalenol. The basis of the tests is the antigen-antibody reaction. Clostridial spores were enumerated according to the methodology described by Bergère and Sivelä [22].

For aerobic stability measurement, approximately 600 g of silage was placed into a polystyrene box allowing air ingress. A thermocouple wire was inserted in the middle of the sample and connected to a data logger. Temperature was automatically recorded at 10 min intervals for a 480-h follow-up period. Aerobic stability was defined as the time taken to increase the temperature of the sample by 2 °C above the ambient temperature. The ambient temperature was 22.1 ± 0.43 °C (min. 20.8 °C and max. 22.9 °C), measured using a similar data logger as for the samples.

The total DNA was extracted from 0.25 g of freeze dried and ground silage samples as described by Yu and Morrison [23]. DNA was eluted in 200 µL of Buffer AE from QIAamp DNA Stool Mini Kit (Qiagen; Sollentuna, Sweden) and 2 µL of DNA was run on a 0.8% agarose gel for quality and integrity control. For bacterial amplicon sequencing, universal primers 515F and 806R targeting the 16S rRNA gene V4 region were used [24]. Libraries

were prepared following the "16S metagenomics sequencing library preparation" protocol (Illumina) and sequenced on an Illumina MiSeq platform using 2×250 bp chemistry at the Finnish Functional Genomics Centre (Turku, Finland). Demultiplexing of sequences, adapter removal and sorting sequences by barcode were performed by the sequencing data provider. Sequencing data was further processed using QIIME v 2 [25]. Briefly, quality control, filtering of chimeric reads, and clustering of bacterial sequences into amplicon sequence variants (ASV) were performed using DADA2 [26]. ASVs with less than 10 reads in total were removed. Bacterial ASV taxonomy was assigned using the Silva 138 database [27].

2.4. Statistical Analysis

Experimental data were analyzed using a Mixed procedure (SAS Inc. 2002–2012, Release 9.4; SAS Institute Inc., Cary, NC, USA) in SAS with additive, compaction and contamination as fixed effects and replicates as a random effect. The Univariate procedure was used to test the normal distribution of data through Shapiro-Wilk test. Least squares means and standard error of the means were reported per treatment and differences among treatment means were declared significant at 5% of probability. A pairwise comparison among treatment means was performed using a Tukey's test.

Silage bacterial community alpha diversity was evaluated using Shannon and Simpson diversity indexes as well as observed number of ASVs. To evaluate treatment effect on the changes in silage microbial community structure, between sample diversity was calculated as Bray–Curtis dissimilarities following Hellinger transformation and visualized using principal co-ordinate analysis (PCoA) as implemented in *MicrobiotaProcess* R package [28]. The significance of groups was evaluated by distance-based permutational multivariate analysis of variance (adonis) and defined at $p < 0.05$ level after 999 permutations, as implemented in *vegan* R package [29].

In order to explore the magnitude of associations between bacterial communities and fermentation quality parameters, the variables were ordered based on a hierarchical cluster analysis of a Spearman correlation plot and a heat map originated from two-dimensional display was created to characterize the effects of bacteria species on fermentation quality. Correlation data was filtered so that all genera below 0.01% were left out. This filtering reduced the number of genera from 192 to 24 groups.

3. Results

3.1. Raw Material Composition and Fermentation Quality of Grass Silage

The botanical composition of the forage was 81% timothy and 19% meadow fescue on fresh matter basis. The forage DM content was 346 g/kg (Table 1), which is representative of the practical target level for grass silages in the region. The relatively high in vitro organic matter digestibility (796 g/kg) and CP concentration (156 g/kg DM) indicate that the grass was cut at an early stage of development and would provide highly nutritious feed.

The two compaction levels used in this experiment were 424 kg/m^3 (147 kg DM/m^3) in loose silages and 583 kg/m^3 (202 kg DM/m^3) in tight silages. Different treatments produced different fermentation profiles in final silages (Table 2). There were no effects ($p > 0.05$) of compaction nor contamination with soil on DM, ash and CP concentrations of the silages. The pH was higher ($p < 0.05$) for loose than tight compaction and higher for non-contaminated than for contaminated material. The control treatment resulted in highest pH among additive treatments ($p < 0.05$) followed by SALT and then FA, while the lowest values for pH ($p < 0.05$) were found for LAB treated silages. Non-contaminated silages resulted in higher concentrations of ammonia ($p < 0.05$) than contaminated silages, and additive treated silages showed lower ($p < 0.05$) concentrations of ammonia than the control treatment. Tight compaction resulted in higher lactic acid concentrations ($p < 0.05$) than loose compaction. The WSC content of the current material was relatively high and use of LAB increased ($p < 0.05$) the conversion of WSC into lactic acid, which may have a positive effect on silage hygienic quality. On the other hand, FA restricted fermentation

resulting in silages with high residual WSC concentration and reduced concentration of total fermentation products.

Table 1. Chemical composition and microbial quality of the mixed timothy and meadow fescue grass prior to ensiling.

Item	
Dry matter (DM), g/kg	346
Buffering capacity, g lactic acid/100 g DM	6.2
Metabolizable energy, MJ/kg DM	11.7
In vitro organic matter digestibility, g/kg organic matter	796
Fermentation coefficient	52.3
In DM, g/kg	
Ash	79
Crude protein	156
Water soluble carbohydrates	137
Neutral detergent fiber	503
Microbial quality	
Yeasts, cfu/g	5.4×10^5
Molds, cfu/g	3.6×10^5
Total bacteria, cfu/g	5.7×10^7
Clostridia, spores/g	<3

cfu: colony-forming unit, estimates the number of viable bacteria or fungal cells in a sample.

Soil contamination increased the amount of acetic acid in silages ($p < 0.01$), but this was due to increases in Control and SALT treatments only. The total concentration of fermentation acids was highest in LAB silages followed by Control and SALT and then FA. Contamination greatly stimulated fermentation acid production in Control and SALT treatments. Compaction did not affect the average level of ammonia N in silages. Use of silage additives decreased the concentration of ammonia N in silages compared to Control silages except for SALT with contamination, which was similar to Control with contamination. Production of butyric and propionic acids was relatively low in all silages.

Residual WSC in silage varied between 5 and 195 g/kg DM (Table 2; in Control, tight, contaminated and in FA, tight, non-contaminated, respectively) and on average was lower in contaminated silages ($p < 0.01$) compared to non-contaminated silages. However, there were no differences in residual WSC in LAB and FA with different compaction or contamination treatments. In all categories FA preserved WSC better than other treatments.

Compaction did not affect aerobic stability, but contamination improved it ($p < 0.01$), because aerobic stability of contaminated Control and SALT silages was relatively high (Table 2). SALT with contamination even remained unheated until the end of the 480-h assessment period. In non-contaminated silages, the ensiling losses were higher in Control and SALT treated silages than in FA and LAB treated silages ($p < 0.05$). In contaminated silages, only FA was able to limit the ensiling losses ($p < 0.05$).

Yeast, mold and clostridia numbers were determined with plate cultivation methods. No significant differences were found in yeast concentrations between compaction or contamination treatments (Table 2). Control silages had numerically higher counts of yeasts than additive treated silages. Contaminated silages had lower concentrations of molds than non-contaminated silages ($p < 0.01$) and no differences between levels of compaction were found. Overall, the numbers of yeasts and molds were relatively low. Clostridial spores were present only in small numbers and the highest abundances were found in non-contaminated tight Control (42 spores/g) and FA (34 spores/g). Mycotoxins were only analyzed from Control and FA silages, and although not statistically significant, zearalenone was five times higher and deoxynivalenol two times higher in Control compared to FA in contaminated silages.

Table 2. Fermentation quality, chemical composition, aerobic stability, ensiling losses and microbial composition of grass silages treated with different additives under two compaction (Comp) and soil contamination (Soil) levels.

Contamination	Non-Contaminated								Soil Contaminated				SEM [1]	p-Value [2]	
Compaction	Loose				Tight				Tight						
Additive	CONT	FA	LAB	SALT	CONT	FA	LAB	SALT	CONT	FA	LAB	SALT		Comp	Soil
Dry matter (DM), g/kg	331 [b]	345 [ab]	348 [ab]	337 [ab]	332 [ab]	345 [ab]	344 [ab]	335 [ab]	341 [ab]	348 [a]	339 [ab]	341 [ab]	3.2	0.67	0.20
pH	5.78 [a]	4.85 [c]	4.02 [ef]	5.48 [b]	5.53 [b]	4.78 [c]	4.00 [f]	5.36 [b]	4.26 [d]	4.83 [c]	4.01 [f]	4.21 [de]	0.039	<0.01	<0.01
Ammonia N, g/kg N	64 [a]	26 [c]	21 [c]	42 [b]	59 [a]	25 [c]	21 [c]	43 [b]	43 [b]	25 [c]	22 [c]	43 [b]	2.2	0.46	0.03
Ash	88 [a]	82 [dc]	85 [abcd]	88 [a]	86 [ab]	82 [d]	85 [abcd]	86 [ab]	86 [ab]	83 [bcd]	84 [bcd]	86 [abc]	0.8	0.11	0.80
Crude protein	177 [a]	164 [c]	172 [abc]	177 [a]	172 [abc]	166 [bc]	171 [abc]	175 [ab]	170 [abc]	169 [abc]	170 [abc]	171 [abc]	1.9	0.27	0.35
Water soluble carbohydrates	87 [cd]	187 [a]	73 [d]	120 [bc]	120 [bc]	195 [a]	76 [d]	135 [b]	5 [e]	181 [a]	66 [d]	6 [e]	8.2	0.02	<0.01
Ethanol	29.9 [ab]	7.8 [de]	3.6 [e]	31.8 [a]	16.5 [c]	4.7 [e]	3.2 [e]	22.0 [bc]	16.4 [c]	2.5 [e]	3.9 [e]	15.0 [cd]	1.56	<0.01	0.06
Silage DM intake index (2007)	120.4 [a]	120.9 [a]	110.0 [cd]	120.6 [a]	120.5 [a]	121.0 [a]	109.8 [d]	120.6 [a]	110.9 [bc]	121.0 [a]	109.8 [d]	111.3 [b]	0.18	0.66	<0.01
Formic	0	8.7	0	0	0	9.5	0	0	0	10.6	0	0	0.14	-	-
Lactic (LA)	12.7 [d]	1.2 [e]	113.1 [a]	12.4 [d]	21.8 [c]	1.6 [e]	114.5 [a]	17.7 [c]	86.2 [b]	0.7 [e]	115.5 [a]	87.8 [b]	0.93	<0.01	<0.01
Acetic	7.5 [c]	7.5 [c]	12.8 [b]	9.0 [bc]	8.3 [bc]	7.7 [c]	12.0 [bc]	9.9 [bc]	30.4 [a]	7.4 [c]	9.4 [bc]	25.9 [a]	0.95	0.72	<0.01
Propionic	0.15 [c]	3.13 [b]	0.08 [c]	0.11 [c]	0.17 [c]	3.45 [a]	0.10 [c]	0.09 [c]	0.24 [c]	2.96 [b]	0.11 [c]	0.23 [c]	0.047	0.02	0.06
Propionic [3]	0.15	0	0.08	0.11	0.17	0	0.10	0.09	0.24	0.34	0.11	0.23	0.100	0.94	0.06
Butyric	0.91	0.28	0.03	0.19	0.29	0.37	0.05	0.16	0.03	0.85	0.03	0.03	0.288	0.51	0.94
Total volatile fatty acids (VFA)	8.64 [bc]	7.82 [c]	12.90 [b]	9.39 [bc]	8.76 [bc]	8.12 [bc]	12.12 [bc]	10.19 [bc]	30.76 [a]	8.82 [bc]	9.55 [bc]	26.18 [a]	0.964	0.87	<0.01
Total fermentation acids [4]	21.3 [d]	9.0 [e]	126.0 [a]	21.8 [d]	30.5 [c]	9.7 [e]	126.6 [a]	27.9 [c]	116.9 [b]	9.5 [e]	125.0 [a]	114.0 [b]	1.16	<0.01	<0.01
LA/total fermentation acids	0.59 [d]	0.12 [e]	0.90 [a]	0.57 [d]	0.71 [bc]	0.15 [e]	0.91 [a]	0.63 [cd]	0.74 [bc]	0.07 [e]	0.92 [a]	0.77 [b]	0.022	<0.01	0.12
Total fermentation products [5]	51 [b]	17 [c]	130 [a]	54 [b]	47 [b]	14 [c]	130 [a]	50 [b]	133 [a]	12 [c]	129 [a]	129 [a]	1.7	0.05	<0.01
Aerobic stability (2 °C), hours [6]	41 [d]	109 [bcd]	118 [bc]	46 [cd]	73 [bcd]	98 [bcd]	133 [b]	48 [cd]	469 [a]	127 [b]	90 [bcd]	480 [a*]	14.8	0.37	<0.01
Ensiling losses, g/kg of initial DM	89 [a]	13 [fg]	3 [i]	60 [d]	79 [b]	10 [gh]	17 [f]	68 [c]	44 [e]	4 [hi]	41 [e]	43 [e]	1.2	0.01	<0.01
Yeasts, cfu/g	4.7×10^5	2.9×10^3	1.6×10^3	1.4×10^4	1.4×10^4	4.3×10^2	3.0×10^2	1.3×10^3	1.0×10^2	9.6×10^2	4.0×10^4	1.0×10^2	9.4×10^4	0.09	0.93
Molds, cfu/g	3.1×10^3 [b]	2.2×10^3 [b]	3.2×10^2 [b]	1.4×10^4 [a]	5.2×10^3 [b]	4.1×10^2 [b]	3.1×10^2 [b]	1.4×10^4 [a]	1.0×10^2 [b]	3.1×10^3 [b]	4.6×10^2 [b]	3.0×10^2 [b]	1.6×10^3	0.94	<0.01
Clostridia, spores/g	na	na	na	na	42	34	3	7	3	13	3	14	16.3	-	0.28
Zearalenone, ppb	403	371	na	na	234	221	na	na	1598	313	na	na	-	-	-
Deoxynivalenol, ppb	299	297	na	na	322	385	na	na	558	252	na	na	-	-	-

CONT: Control, FA: Formic acid-based additive, LAB: Lactic acid bacteria additive, SALT: Salt based additive. Values with same letter in a row are not significantly different at 5% Tukey test. If there are no differences in Tukey test, letters are removed. [1] Standard error of the mean. [2] Effect of compactions and soil contamination. [3] Corrected for its amount in the FA based additive. [4] Total VFA + lactic acid. [5] Total fermentation acids + ethanol. [6] Time taken to increase the temperature of samples by 2 °C above the ambient temperature (22 °C). * Treatment did not reach the threshold during the evaluation period. cfu: colony-forming unit, estimates the number of viable bacteria or fungal cells in a sample. na: not analyzed.

3.2. Bacterial Communities in Grass Silage

After quality filtering, the sequencing data contained 856,959 sequences in total with a mean number of 23,161 ± 12,978 reads per sample. For subsequent analyses the data was evenly subsampled to 14,000 reads per sample.

There were effects of treatments on microbial alpha diversity estimates (Table 3), except for the compaction effect. Soil contaminated silage resulted in lower microbial alpha diversity than non-contaminated silages. Additionally, LAB treated silages produced lower microbial alpha diversity than other additive treatments.

Table 3. Alpha diversity estimates of grass silage treated with different additives under two compaction (Comp) and soil contamination (Soil) levels.

Contamination	Non-Contaminated								Soil Contaminated				SEM [1]	*p*-Value [2]	
Compaction	Loose				Tight				Tight						
Additive	CONT	FA	LAB	SALT	CONT	FA	LAB	SALT	CONT	FA	LAB	SALT		Comp	Soil
Observed ASV [3]	248 ab	205 abc	118 bc	317 a	231 abc	203 abc	161 bc	244 ab	101 bc	232 abc	124 bc	83 c	30.0	0.57	<0.01
Shannon	5.12 a	4.90 a	3.51 b	5.16 a	5.10 a	4.89 a	3.58 b	5.07 a	2.91 c	5.02 a	3.45 b	2.87 c	0.099	0.86	<0.01
Simpson	0.991 a	0.989 a	0.939 b	0.990 a	0.992 a	0.989 a	0.939 b	0.990 a	0.898 c	0.990 a	0.933 b	0.898 c	0.0035	0.95	<0.01

CONT: Control, FA: Formic acid-based additive, LAB: Lactic acid bacteria additive, SALT: Salt based additive. Values with same letter in a row are not significantly different at 5% Tukey test. [1] Standard error of the mean. [2] Effect of compactions and soil contamination. [3] Observed amplicon sequence variants.

Silage bacterial community structure was significantly (adonis test *p* < 0.001) affected by treatment (Figure 1). Raw material sample before ensiling was identified apart from the silages. LAB treated silages across compaction and contamination treatments formed their own cluster. Similarly, Control and SALT when contaminated under tight compaction also formed their own cluster, while the remaining silages clustered in a separate group.
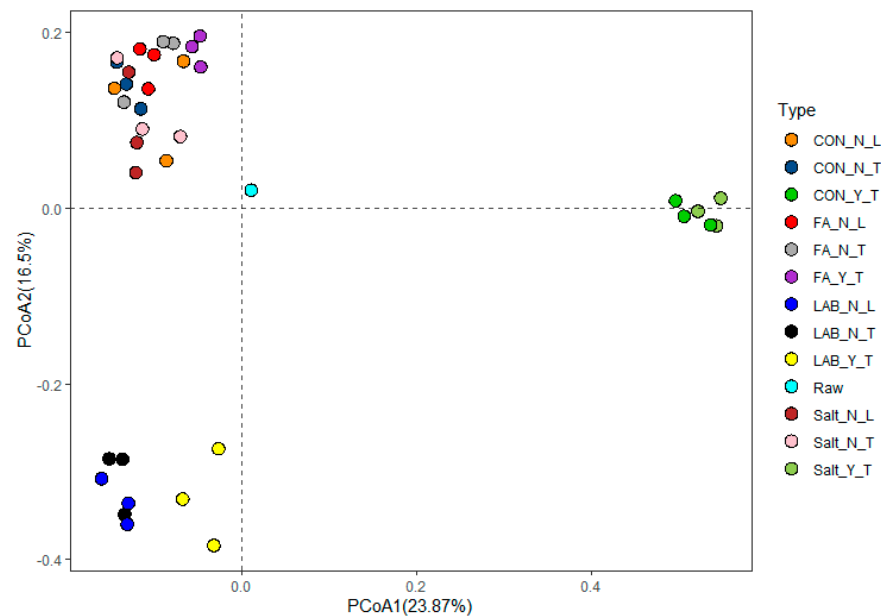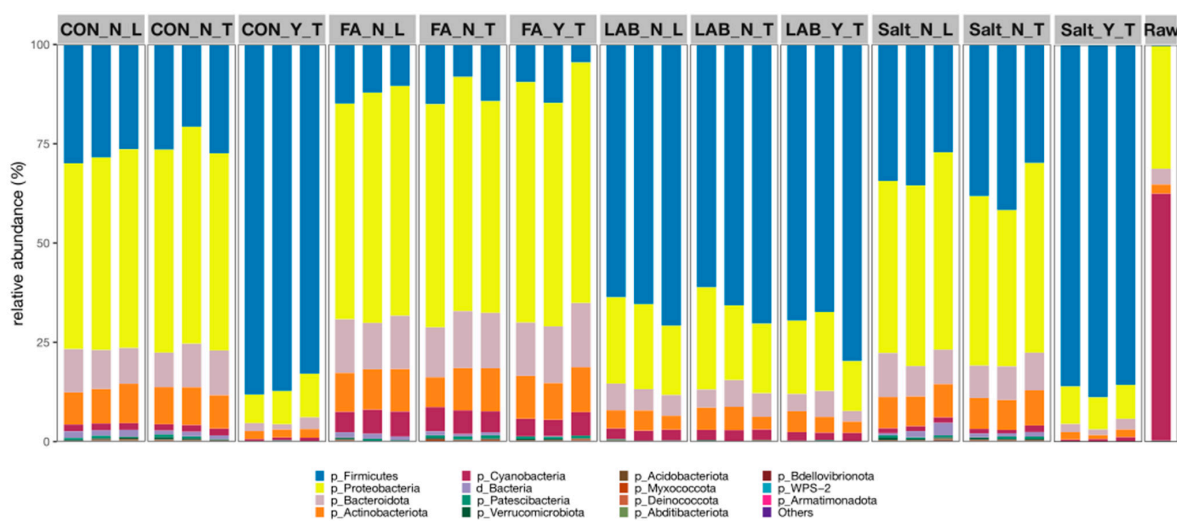

Figure 1. Beta diversity analysis of grass silage treated with different additives under two compaction and soil contamination levels. CON: Control, FA: Formic acid-based additive, LAB: Lactic acid bacteria additive, Salt: Salt based additive, N: non-contaminated, Y: contaminated, L: loose compaction, T: tight compaction, Raw: raw material before ensiling.

There were clear differences in the relative abundance of bacterial communities according to the different treatments (Figure 2). In the total data set 16 bacterial phyla were observed. The most abundant phyla were Firmicutes, Proteobacteria and Bacteroidota. These phyla were represented by 130 bacterial genera in total; however, the top 40 abundant genera are presented. Firmicutes phylum dominated the microflora of LAB treated silages (Figure 2A). Additionally, Firmicutes also dominated the microbial fermentation profiles of Control and SALT treated silages under contamination and tight compaction. Proteobacteria phylum was the second largest community in LAB silages, while it dominated FA treaded silages along with Control and SALT under non-contamination, and for both tight and loose compactions. Ensiling caused a clear compositional shift in microbial populations as the epiphytic community in fresh raw material before ensiling was mainly dominated by Cyanobacteria phylum, followed by Proteobacteria.
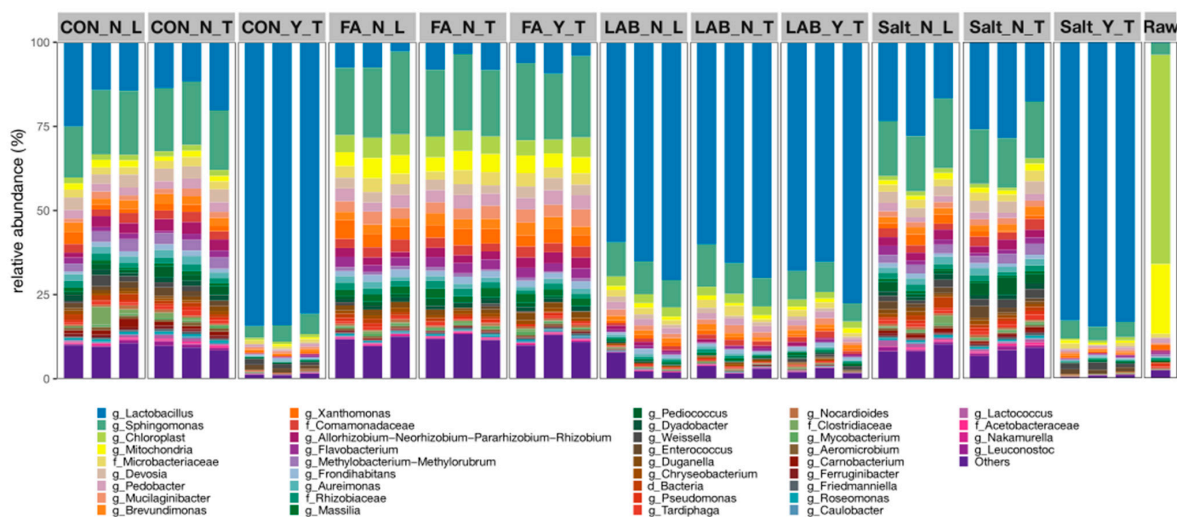
(A)

(B)

Figure 2. Relative abundance of bacterial communities of grass silage treated with different additives under two compaction and soil contamination levels. (**A**): Phylum taxonomic level, (**B**): Genus taxonomic level, CON: Control, FA: Formic acid-based additive, LAB: Lactic acid bacteria additive, Salt: Salt based additive, N: non-contaminated, Y: contaminated, L: loose compaction, T: tight compaction, Raw: raw material before ensiling.

The silage fermentation microflora was in large proportion dominated by the genus *Lactobacillus* for LAB treated silages, along with Control and SALT when grass was contaminated and under tight compaction (Figure 2B). *Sphingomonas* was the most prevalent genus dominating fermentation of FA silages. Formic acid led to much more diverse populations, even with contamination than other additive treatments. Contamination of raw material did not affect the bacterial composition of FA-treated silages. *Lactobacillus* and *Sphingomonas* genera were equally present in Control and SALT treated silages in non-contaminated grass, and for both tight and loose compactions.

Spearman's correlation analysis was performed to identify the relationships between bacterial genera and fermentation quality parameters of the non-contaminated silages (Figure 3). Strong positive correlations were found between pH, ammonia N and ethanol, and many communities, especially the genera *Carnobacterium*, *Methylobacterium-Methylorubrum* and *Devosia*. Only the *Lactobacillus* genus was clearly and significantly correlated with the decrease of pH and ammonia N. *Lactobacillus* had a strong positive correlation with most of the fermentation quality parameters evaluated in silage, such as lactic acid, acetic acid, total VFA, total fermentation acids, total fermentation products and lactic acid:total fermentation acids ratio. *Sphingomonas*, *Duganella*, *Massilia* and *Xanthomonas* were negatively correlated with the fermentation acids of the silages, such as lactic and acetic acids, and consequently with total VFA, total fermentation acids and total fermentation products. *Mucilaginibacter* was negatively correlated with ensiling losses.

	pH	Ammonia N	Ash	Crude Protein	WSC	Ethanol	Lactic Acid	Acetic Acid	Propionic Acid	Butyric Acid	Total VFA	TFA	LA/TFA	TFP	Aerobic Stability	Ensiling losses
Microbacteriaceae			−0.50	−0.61	0.78		−0.55	−0.56	−0.53		−0.69	−0.58	−0.56	−0.74		
Frondihabitans			−0.58	−0.67	0.61		−0.61	−0.68			−0.72	−0.62	−0.62	−0.71		
Dyadobacter	0.76	0.70			0.47	0.63	−0.48	−0.52			−0.48	−0.49	−0.48	−0.53	−0.69	0.54
Flavobacterium				−0.10	0.85		−0.81	−0.67	−0.45		−0.79	−0.83	−0.82	−0.88		
Chryseobacterium	0.31				0.72		−0.65	−0.44			−0.56	−0.64	−0.67	−0.63		
Mucilaginibacter			−0.57	−0.61	0.70		−0.57	−0.48	−0.60		−0.53	−0.57	−0.58	−0.67		−0.31
Pedobacter			−0.35	−0.51	0.65		−0.59	−0.66	−0.45		−0.74	−0.63	−0.55	−0.77		
Carnobacterium	0.88	0.89	0.60	0.44		0.88	−0.28	−0.45	0.56		−0.32	−0.28			−0.75	0.81
Enterococcus	0.79	0.77	0.70	0.61		0.78			0.60						−0.62	0.77
Lactobacillus	−0.38	−0.36			−0.79		0.81	0.88			0.91	0.83	0.79	0.88	0.24	
Pediococcus	0.63	0.69				0.63	−0.21					−0.20		−0.42	−0.40	0.58
Weissella	0.65	0.68	0.44			0.72			0.53	0.45					−0.61	0.69
Brevundimonas	0.43				0.73		−0.69	−0.69			−0.78	−0.72	−0.65	−0.79		
Methylobacterium-Methylorubrum	0.85	0.91	0.49			0.77	−0.20	−0.42	0.52		−0.32	−0.21		−0.32	−0.66	0.83
Devosia	0.80	0.77			0.44	0.60	−0.33	−0.53			−0.52	−0.36	−0.30	−0.52	−0.61	0.70
Rhizobiaceae	0.42	0.43			0.75		−0.64	−0.75			−0.70	−0.64	−0.65	−0.79		
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0.71	0.73			0.44	0.55	−0.37	−0.61			−0.53	−0.37		−0.56	−0.44	0.52
Aureimonas	0.79	0.80				0.70	−0.39	−0.59			−0.55	−0.41		−0.40	−0.59	0.69
Tardiphaga	0.70	0.76				0.71	−0.20	−0.34			−0.27	−0.20		−0.37	−0.58	0.68
Sphingomonas	0.41				0.72		−0.85	−0.87			−0.88	−0.86	−0.83	−0.78		
Comamonadaceae	0.52	0.57			0.60		−0.54	−0.64			−0.69	−0.57	−0.51	−0.68		
Duganella	0.38				0.70		−0.70	−0.65			−0.70	−0.69	−0.74	−0.73		
Massilia			−0.45	−0.58	0.75		−0.67	−0.61	−0.48		−0.70	−0.69	−0.68	−0.79		
Xanthomonas			−0.24	−0.42	0.71		−0.83	−0.75	−0.37		−0.85	−0.82	−0.83	−0.75		

Figure 3. Spearman correlations between bacterial communities and silage parameters of grass silage with different additives. WSC: Water soluble carbohydrates, VFA: Volatile fatty acids, TFA: Total fermentation acids, LA: Lactic acid, TFP: Total fermentation products. From green (strong and positive correlation) to red color (strong and negative correlation), while empty cells indicate not significant correlation.

4. Discussion

4.1. Raw Material Characteristics

The grass DM content was relatively high for only slightly wilted grass due to a very dry weather prior to harvesting. The chemical composition and in vitro organic matter digestibility indicate a nutritionally high-quality material under Northern European conditions [17]. The fermentation coefficient of 52.3 indicates relatively easy ensilability of the material, due to high DM and WSC concentrations in relation to the buffering capacity of the material [20].

4.2. Fermentation Quality

Silage compaction is considered a crucial management factor in practice [30]. Loosely compacted silage was connected to decreased silage quality with corn silage especially at feed-out phase [31] although delayed sealing of silo impacted silage quality even more. Brüning et al. [31] also noted that before sealing the silo, loose compaction (194 kg DM/m^3) caused higher silage temperature than tight compaction (234 kg DM/m^3). The fermentation quality of the loosely compacted silages was in many respects lower that of the tightly compacted silos also in the current study. The magnitude of the difference was however relatively small in line with McEniry et al. [32] using laboratory silos. It is likely that laboratory silos are not able to totally mimic the responses to compaction in farm scale silos, because they may be more airtight, and also they are sampled immediately after opening while oxygen keeps penetrating into the farm scale silos during storage and particularly during the feed-out period. Both compaction levels used in the current study were below the suggested density of 210 kg DM/m^3 [33] as the density was 147 kg DM/m^3 in loosely compacted silos and 202 kg DM/m^3 in tightly compacted silos. Since the effect of compaction was not very large, this is probably not a crucial factor in laboratory scale silos.

Contamination should be avoided in silage making but interestingly, in the current data it seemed to promote lactic acid fermentation and reduced pH in Control and SALT that were otherwise relatively poorly preserved. For some reason, natural epiphytic bacteria were not able to preserve them adequately. However, the wild-type fermentation caused by contamination resulted in very high acetic acid concentration in the silages and poses risks to the hygienic quality of the silages. According to Kung [34], production of acetic acid through wild-type fermentation pathways may be less desirable than if produced via controlled pathways by, e.g., inoculating the silage with selected heterofermentative lactic acid bacteria.

The effects of additives on silage fermentation quality were in general typical [1] and all additives improved the fermentation quality compared to the Control, although SALT was somewhat less effective than FA and LAB. Formic acid almost totally prevented fermentation while that of LAB promoted it in line with, e.g., Seppälä et al. [35]. A high amount of lactic acid is beneficial for ensiling the forage but decreases the voluntary DM intake of silage by animals [21,36].

It is noteworthy that FA and LAB treated silages performed very consistently despite of the compaction or contamination treatments, while control and SALT silages were more variable. Formic acid treatment was able to restrict the fermentation even with contaminated grass and it has been proven to be effective in difficult conditions, such as when preserving very low DM content materials (<200 g/kg FM) [35,37,38]. The residual WSC in FA treated silages was much higher than in other treatments and even higher than in the raw material. The restricted fermentation for FA treated silages resulted in silages with high residual WSC concentration and reduced concentration of total fermentation products, which would be beneficial if WSC content in the grass before ensiling is low and may promote higher intake of silage by animals. These results were consistent across different management factors, such as compaction and contamination, and may be explained by acid hydrolysis of NDF that may have yielded more sugars [1]. The NDF concentration was not analyzed in current silages, but in a data set of 52 formic acid treated grass silages,

the NDF concentration of silages was on average 27 g/kg DM lower than in the parent herbages [39].

4.3. Aerobic Stability

Heating of silage due to the activity of aerobic microbes, initiated typically by yeasts and continued by molds, has become an increasingly important problem as the grass is wilted to higher DM [33]. In our case, compaction did not affect aerobic stability although it is considered an important factor at farm scale. The discrepancy may be related to the laboratory scale silos being more airtight than farm scale silos, and the aerobic stability test being commenced immediately after silo opening so that air-ingress into the silage mass during the feed-out phase was not mimicked. These factors would result in lower counts of yeasts than under practical conditions. Pauly and Wyss [40] concluded that without an appropriate air stress, untreated control silages can sometimes show better aerobic stability than additive treated silages.

Undissociated acetic acid has been proven to be effective in improving silage aerobic stability [41,42]. This may however be less desirable if acetic acid is produced under uncontrolled fermentation pathways [34]. Further, Wilkinson and Davies [33] reviewed that soil-borne microorganisms including enterobacteria (coliform organisms), clostridia and fungi are likely to result in a fermentation type that may improve aerobic stability of the ensiled material. Although seemingly positive from the aerobic stability point of view, this type of fermentation cannot be recommended when looking at ensiling quality as a whole.

Based on the previous discussion, the evaluation of additive effects on silage aerobic stability is challenging. Wilkinson and Davies [33] concluded that in several cases, the use of a homofermentative LAB actually decreases aerobic stability by reducing the acetic acid concentration. In our case, however, the only significant additive effect in non-contaminated silages was the longer aerobic stability of LAB compared to Control in loosely compacted silages. The longer aerobic stability of Control and SALT in contaminated silages is related to the wild-type fermentation that could be controlled by FA and LAB, and thus resulted in a shorter aerobic stability of them.

4.4. Microbial Composition

Soil contamination negatively affected bacterial community diversity. Lower alpha diversity was identified in soil contaminated silages as compared to non-contaminated ones, probably because of the initial inoculation with soil containing specific communities that modulated the silage fermentation. However, compaction did not cause any variation on the diversity of communities. Interestingly, LAB had the lowest alpha diversity of all additive treatments, possibly due to the early-stage inoculation with *Lactobacillus plantarum*. Interestingly, the beta diversity of the fresh forage was identified apart from the silages and clearly distinct clusters were found for the additive treatments.

The most abundant phyla in silage were Firmicutes and Proteobacteria, which varied in their proportion according to the treatments, but still represented the largest communities across all silages. Wang et al. [43] found similar pattern studying silages prepared with mixtures of alfalfa and sainfoin, where the silage microflora was dominated by Firmicutes, followed by Proteobacteria.

In the raw material, aerobic epiphytic bacteria are dominant, but they are suppressed during fermentation when facultative and strictly anaerobic bacteria start to dominate [2,44]. Although LAB-treated silages presented the greatest proportions of *Lactobacillus* genus, which might be because of its inoculation, the species was not fully taxonomically identified. With bacterial inoculants, the added bacterium is often still not the most dominant one in silage and it may just modulate the shift of whole bacterial community during ensiling [45].

Regarding relative abundance of bacterial communities, LAB treated silages resulted in higher proportions of *Lactobacillus*, which could potentially be *Lactobacillus plantarum*, but species-level classification could not be obtained. The inoculation of the Control and SALT

with soil probably had a similar effect, as communities were inserted in the material from the beginning, and the predominant population was also *Lactobacillus*. It is worth mentioning that although most of the populations in these treatments were *Lactobacillus* genus, it is not possible to say that they are the same species. Formic acid had a strong suppressing effect to prevent the proliferation of *Lactobacillus*, even when inoculated with soil.

Relative bacterial abundances of FA and LAB were different from each other, but both remained almost the same even with differences in compaction and contamination. Effective silage additives seem to be able to control the silage microbiome also in challenging ensiling conditions. In Control and SALT silages, the variation was larger than in FA and LAB silages, mostly because the contaminated Control and SALT silages clearly had different microbiomes from any other silages.

It is rather important to emphasize that the identification of microbial composition of fermented silages based on sequencing of 16S rRNA may not differentiate communities still viable and dead bacterial cells [46]. This shortcoming might possibly misrepresent the composition of microbial communities in silages.

4.5. Correlations between Bacterial Communities and Silage Characteristics

The role of several bacterial communities on different ecosystems is well known [47]. However, the correlation between different bacterial communities and the silage fermentation ecosystem is still not well defined.

Correlation between fermentation parameters and bacterial abundance could mean that bacteria promoted or restricted the amount of fermentation product, or the other way. For example, the strong negative correlation between lactic acid and *Dyadobacter*, *Chryseobacterium*, *Carnobacterium*, *Pediococcus*, *Sphingomonas* and *Duganella* more likely means that these communities are associated with poor fermentation quality, which consequently resulted in high pH of silages, evidenced by the strong positive correlation with those same communities. An opposite example is a positive correlation between genera *Carnobacterium*, *Enterococcus*, *Weissella*, *Methylobacterium-Methylorubrum* and *Tardiphaga*, and ethanol, which probably indicate that those bacteria promote the production of ethanol in silage. Ethanol, which is undesirable in ensiling, could be produced by these bacteria or by some other microbe(s) having positive correlation with them. The strong positive correlation of *Enterococcus* genus with ammonia suggests that this genus increased protein losses even though some species, such as *Enterococcus faecium*, are widely used as inoculant additives. Diversity within one genus can be remarkable and species-level information is needed when bacteria are selected to be used as inoculant additives.

The longer the aerobic stability of a silage, the better, as it means that the silage remains stable to be fed to animals. However, the few correlations identified between aerobic stability and different genera were negative, which implies on shorter aerobic stability when *Dyadobacter*, *Carnobacterium*, *Enterococcus*, *Weissella*, *Methylobacterium-Methylorubrum* and *Aureimonas* genera are abundant in silage.

Butyric acid was the fermentation quality parameter least statistically significantly correlated with bacterial communities in silage. The only positive correlation of butyric acid was found with *Weissella*, which might indicate that *Weissella* is strongly correlated to poor fermentation, given that butyric acid is an undesirable acid found in silages [48].

Species from *Lactobacillus* and *Pediococcus* genera are commonly used as silage additives [44]. Both are within *Leuconostocaceae* family, however the first was correlated with fermentation parameters related to strong and favourable fermentation, while the second was associated to poor fermentation quality. Residual WSC was also negatively correlated with *Lactobacillus*.

Ogunade et al. [49] scanned bacterial communities in high DM alfalfa silage and found that *Sphingomonas* correlated with fermentation quality parameters in a beneficial way for silage preservation. The genus had negative correlation with silage pH, ammonia-N and growth of molds and yeasts. However, in the present data, *Sphingomonas* correlated

positively with silage pH and negatively with lactic acid production, which is opposite to findings of Ogunade et al. [49].

Interestingly, after data filtration, most of the remaining bacterial communities used in the correlation analyses were those correlated to poor fermentation quality of silages, except the *Lactobacillus* genus. *Lactobacillus*, among all communities, was the only one showing correlations to an ideal fermentation quality, provided that it was positively correlated to lactic acid and total fermentation acids production, and at the same time, negatively correlated to pH and ammonia N. It has been shown that the *Lactobacillus* genus, belonging to the Firmicutes phylum, is linked to desirable lactic acid fermentation [49,50]. This genus is also widely used in silage additives. In the meta-analysis of Oliveira et al. [51], 67% of experimental silages were preserved with *Lactobacillus plantarum* and 27% with a mixture of two or more lactic acid bacteria species. The strong positive correlation of the *Lactobacillus* genus with both acetic acid and aerobic stability indicates the predisposition of this genus to improve the aerobic stability of silages through boosting acetic acid production. Some heterofermentative *Lactobacillus* species such as *Lactobacillus buchneri* are used as silage additives, because they can produce acetic acid and subsequently improve aerobic stability [33,41,52].

5. Conclusions

The use of formic acid, selected lactic acid bacteria strains and salt-based additives improved fermentation quality of grass ensiled under different management conditions. Tight compaction resulted in well preserved silages and should be aimed at in farm scale. Contamination stimulated wild-type fermentation that is not recommended as an ideal pathway to preserve silage under farm conditions, because it could cause losses in nutritive value and hygienic risks in the food chain even though it showed some positive effects in fermentation quality and improved the aerobic stability of silages.

Great shift was observed in bacterial profiles from fresh material towards silage. *Lactobacillus* genus was barely found on the relative abundance of fresh grass but became predominant in the final silage along with *Sphingomonas* genus. Different types of additives modulated the bacterial profiles of the silages. Strong correlations between bacterial communities and fermentation quality parameters provided clear insight of the role of the most abundant populations on the fermentation process of grass silage.

Associations between silage quality parameters and silage bacterial communities could be used when new silage additives are investigated. This approach allows evaluation of the role of the most abundant bacterial populations in the fermentation process of silage and which bacteria have the greatest impact on each fermentation parameter. More detailed knowledge is needed all the way to species-level to select new bacterial silage additives. More information is needed about microbiome changes in silages made from variable raw materials including forage legumes.

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