University of Kent

Kent Academic Repository

Filippova, Valentina A., Ilina, Larisa A., Yildirim, Elena A., Dubrovin, Andrei V., Ponomareva, Ekaterina S., Dubrovina, Alisa S., Klyuchnikova, Irina A., Zaikin, Vasiliy A., Malakhov, Ivan G., Laptev, Georgi Yu. and others (2026) ***Effectiveness evaluation of microbiological preparations for preserving ensiled plant feeds in a model experiment using microbiomic and bioinformatic tools.*** **In: Digital Technologies in Aquaculture, Organic Farms and Dairy Productions Proceedings of the Fifth International Conference on Agriculture Digitalization and Organic Production (ADOP 2025), Volume 2. Smart Innovation, Systems and Technologies , pp. 335-346. Springer, Cham, Switzerland ISBN 978-3-032-17406-2. E-ISBN 978-3-032-17407-9.**

Downloaded from
https://kar.kent.ac.uk/115818/ The University of Kent's Academic Repository KAR

The version of record is available from
https://doi.org/10.1007/978-3-032-17407-9_29

This document version
Author's Accepted Manuscript

DOI for this version

Licence for this version
CC BY (Attribution)

Additional information
For the purpose of open access, the author(s) has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising.

Versions of research works

Versions of Record
If this version is the version of record, it is the same as the published version available on the publisher's web site. Cite as the published version.

Author Accepted Manuscripts
If this document is identified as the Author Accepted Manuscript it is the version after peer review but before type setting, copy editing or publisher branding. Cite as Surname, Initial. (Year) 'Title of article'. To be published in ***Title of Journal*** , Volume and issue numbers [peer-reviewed accepted version]. Available at: DOI or URL (Accessed: date).

Enquiries
If you have questions about this document contact ResearchSupport@kent.ac.uk. Please include the URL of the record in KAR. If you believe that your, or a third party's rights have been compromised through this document please see our Take Down policy (available from https://www.kent.ac.uk/guides/kar-the-kent-academic-repository#policies).

Effectiveness Evaluation of Microbiological Preparations for Preserving Ensiled Plant Feeds in a Model Experiment Using Microbiomic and Bioinformatic Tools

Valentina A. Filippova[1], Larisa A. Ilina[1,2], Elena A. Yildirim[1,2], Andrei V. Dubrovin[3], Kseniya A. Sokolova[1], Ekaterina S. Ponomareva[1], Alisa S. Dubrovina[1], Irina A. Klyuchnikova[1], Vasiliy A. Zaikin[1], Ivan G. Malakhov[2], Georgi Yu. Laptev[1], Darren K. Griffin[4,5], Michael N. Romanov[2,4,5,6]*

[1]Molecular Genetics and Microbiomics Laboratory, BIOTROF+ Ltd, Pushkin, St. Petersburg, 196602, Russia
[2]Federal State Budgetary Educational Institution of Higher Education "St. Petersburg State Agrarian University", Pushkin, St. Petersburg, 196605, Russia
[3]Information Technologies, Mechanics and Optics (ITMO) University, St. Petersburg, Russia
[4]School of Natural Sciences, University of Kent, Canterbury, Kent, CT2 7NJ, UK

[5]Animal Genomics and Bioresource Research Unit (AGB Research Unit), Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand

[6]L. K. Ernst Federal Research Center for Animal Husbandry, Dubrovitsy, Podolsk, Moscow Oblast, 142132, Russia

Abstract Ensiling is the main method of preparing bulk forages for cattle in the conditions of the risk farming zone. This zone includes St. Petersburg and the Leningrad Oblast due to their geographical location and high humidity. To improve the efficiency of enzymatic processes during ensiling, various biopreparations of lactic acid bacteria that consist of one or more strains are used. However, the biotechnological potential of lactic acid bacteria involved in silage fermentation remains insufficiently studied. Thus, the selection of microorganisms for use in silages should always be carried out with all rigor and meet

certain criteria. The aim of this study using metagenomic next-generation sequencing (NGS), endotoxin genes analysis and bioinformatics was to assess the efficiency of applying monocultures of lactic acid bacteria strains (*Lactobacillus plantarum* 50 and *Enterococcus faecium* 46). We further evaluated combining these strains for the ensiling process in a model laboratory experiment. As a result, it was shown that the greatest stability of microbiome and a high proportion of lactobacilli in the ensiled feeds, the best pH levels and silage quality were achieved using a combination of strains (*L. plantarum* 50 + *E. faecium* 46). Such a strategy is most promising for new drug production, since it has a synergistic effect from the activities of two complementary strains of lactic acid bacteria. Its use also helps protect animals from pathogenic silage bacteria (clostridia and enterobacteria), as well as their endotoxins, and helps secure food products to be safe for human consumption.

Keywords. Biopreparations, strains, bacteria, plant feed, ensiled feeds, microbiome, metagenomic next-generation sequencing (NGS), endotoxin genes, bioinformatics

1. Introduction

Ensiling is one of the most well-known methods of feed preservation. Silage is a vital component of the diet of farm ruminant animals. It typically consists of fermented plant material stored in a sealed environment with high moisture content (e.g., [1]). The rapid development of livestock production increases the demand for silage [2]. Ensilage production is especially important in areas where winters are long and cold, and animals cannot obtain the energy or nutrients they need from grazing year-round. This is particularly relevant in countries with humid climates where hay production and storage are difficult [3].

Silage fermentation requires anaerobic conditions that suppress the development of aerobic microorganisms such as aerobic bacteria, yeasts, and molds that can cause spoilage of plant material [4]. Fermentation of plant biomass occurs naturally under anaerobic

conditions, which is explained by the predominance of bacteria on the plant surface. Epiphytic lactic acid bacteria use simple carbohydrates present in plants, metabolizing them to lactic acid and, to a lesser extent, to acetic acid. These processes are able to prevent silage spoilage and ensure the preservation of silage for a long time [5]. However, the frequency and efficiency of fermentation in terms of pH reduction are highly dependent on the number and type of lactic acid bacteria present in plant biomass, which can suppress the activity of undesirable bacteria [6]. Natural populations of lactic acid bacteria on plant raw materials are often heterofermentative and few in number [7], meaning that epiphytic bacterial microflora is often insufficient to initiate the formation of lactic acid in large quantities. Therefore, obtaining silage of good quality and high digestibility requires stimulation of the ensiling process by adding various biopreparations (e.g., [1,8]). One of the safest methods of silage preservation is lactic acid bacteria-enhanced fermentation [9].

The introduction of different strains of lactic acid bacteria significantly affects the microbial composition of silage [10]. Lactic acid bacteria are added to prevent the development of spoilage microorganisms, quickly reduce silage pH, and increase dry matter recovery [11]. Inoculated microorganisms also reduce the activity of pathogenic microorganisms [12]. Bacteria of the genus *Lactobacillus* are known to suppress the proliferation of other microorganisms effectively [13]. During the initial fermentation stage, lactic acid bacteria protect feed from spoilage microorganisms by producing beneficial organic acids and antifungal agents such as bacteriocins [9].

Silages inoculated with lactic acid bacteria have and maintain a stable pH, resulting in acidic conditions that limit microbial activity and population, and potentially harmful species such as Clostridia and Bacilli can live as spores as long as the silage remains sealed [14]. Furthermore, several studies have reported the ability of lactic acid bacteria to maintain lower silage pH, beneficial organic acids and metabolites, and reduced pathogenic activity after silage opening, ensuring high aerobic stability [15–17].

To improve the efficiency of enzymatic processes during silage making, it is proposed to use the synergistic effects of combining several types of lactic acid bacteria [18]. Currently, studies have been published on combining lactic acid bacteria to improve silage quality with successful results [16]. Treatment of plant biomass with

combined inoculants reduces the activity of yeast and mold, as well as other aerobic bacteria that use lactic acid produced in sufficient quantities by homofermentative lactic acid bacteria [19,20].

Previously, we examined metagenomic composition, gene expression and endotoxins in feed and body microbiomes of lactating cows [21–23]. Consequently, following on from these previous experiments, the aim of this study was to determine the effectiveness of using monocultures of lactic acid bacteria strains (*Lactobacillus plantarum* 50 and *Enterococcus faecium* 46). We further combined these strains for the ensiling process in a model laboratory experiment using microbiomic and bioinformatic approaches.

2. Materials and Methods

2.1. Setting up a model laboratory experiment on silage preservation

The laboratory experiment was conducted on a cereal-legume mixture in the budding phase, common in the Leningrad Oblast, Russia. The experimental design and the quality assessment of feed supplemented with biopreparations were based on the methodological recommendations for conducting experiments on the preservation and storage of bulk feed [24]. In the experiment, four variants of silage treatment were employed: sample No. 1, no treatment (control) variant, i.e., without the use of microorganism strains; sample No. 2, using two strains of lactic acid bacteria (*L. plantarum* 50 and *E. faecium* 46); sample No. 3, using the strain *L. plantarum* 50; and sample No. 4, using the strain *E. faecium* 46 alone. Laboratory experiments on the ensiling of bulk feed were executed using vacuum bags in three replicates. For this, green mass weighing 300 g, 500 g and 1 kg was placed in bags, then air was pumped out of them and simultaneously the bags were sealed using a chamberless vacuum packer Lava V.333 Premium. The result was a compressed mass in an airless space. The bags with silage were placed in a thermostatic room at 36 ± 1 °C.

2.2. Analysis of biochemical composition and nutritional value of feed

Silage samples for biochemical and nutritional analyses were collected on Day 30. The following indicators were determined in the

feed samples: dry matter by drying the samples at 105 °C (in accordance with GOST 31640-2012), crude protein (according to GOST 13496.4-2019), digestible protein (following GOST R 51423-99), crude fiber using Henneberg and Stohmann method (according to GOST 31675-2012), crude fat using Soxhlet method (following GOST 13496.15-2016), sugar content using Bertrand method (according to GOST 26176-2019), crude ash (following GOST 26226-95), acid detergent fiber (ADF) content (following GOST ISO 13906-2013), neutral detergent fiber (NDF) content (according to GOST ISO 16472-2014), mass fractions of lactic, acetic and butyric acid (following GOST R 55986-2022), content of feed units (according to GOST 23630-90), and metabolizable energy (ME) according to the method for calculating ME in feed (L. K. Ernst Federal Science Center for Animal Husbandry, Dubrovitsy, Russia, 2008). Silage samples for ensiling process pH control were taken dynamically, i.e. on Days 1, 2, 3, 5, 8, 15 and 30. Hereby, active acidity level (pH) was measured according to GOST 26180-84.

2.3. Detection of bacterial endotoxin genes using PCR method

Using PCR, the samples were tested for the presence of genes for Shiga toxins (*stx1A* and *stx2B*), intimin (*eae*) and enterohemolysin (*ehxA*) produced by enterobacteria (*Escherichia coli*); alpha toxin (*cpa1*), beta toxin (*cpb*) and epsilon toxin (*etx*) produced by *Clostridium perfringens*; and binary toxin (*cdtB*) and toxins A (*tcdA*) and B (*tcdB*) produced by *C. difficile* (e.g., [23]). Primers for the detection of endotoxin genes were designed using the NCBI Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The housekeeping gene *Eub339-518* (16S rRNA) was used as a control. Amplification reactions were performed using a DTlite amplifier (DNA-Technology, LLC, Moscow, Russia) and a Tersus Plus PCR kit (ZAO Evrogen, Russia) according to the manufacturer's protocol. The PCR amplification mode and conditions were as follows: 3 min at 94 °C (preheating), 40 s at 94 °C, 60 s at 55 °C, and 90 s at 72 °C (34 cycles). Amplified gene fragments were analyzed by gel electrophoresis for the presence of a band of the expected size.

Table 1 Sequences of primers used in PCR study of endotoxin genes

Gene	Primer sequences (5′–3′)	Amplicon length, bp
stx1A	F:CCTTTCCAGGTACAACAGCGGTT R:GGAAACTCATCAGATGCCATTCTGG	478
stx2B	F:AAATATGAAGAAGATATTTGTAGCGGC R:CAGCAAATCCTGAACCTGACG	251
eae	F:GGAACGGCAGAGGTTAATCTGCAG R:GGCGCTCATCA TAGTCTTTC	346
ehxA	F:GCATCATCAAGCGTACGTTCC R:AATGAGCCAAGCTGGTTAAGCT	534
cpa1	F:GCTAATGTTACTGCCGTTGA R:CCTCTGATACATCGTGTAAG	324
cpb	F:TCCTTTCTTGAGGGAGGATAAA R:TGAACCTCCTATTTTGTATCCCA	611
etx	F:TGGGAACTTCGATACAAGCA R:TTAACTCATCTCCCATAACTGCAC	396
cdtB	F:TTGACCCAAAGTTGATGTCTGATTG R:CGGATCTCTTGCTTCAGTCTTTATAG	262
tcdA	F:GCATGATAAGGCAACTTCAGTGGTA R:AGTTCCTCCTGCTCCATCAAATG	629
tcdB	F:CCAAARTGGAGTGTTACAAACAGGTG R:GCATTTCTCCATTCTCAGCAAAGTA	410

2.4. Investigating the silo microbiome using sequencing and bioinformatics

To study the microbial community of silos, high-throughput metagenomic next-generation sequencing (NGS) based on a fragment of the 16S rRNA gene was used, which allows for the metagenomic composition of the microbiota and taxonomic affiliation of its identified representatives to be characterized with sufficient accuracy (e.g., [1,7,23]). Samples were collected under aseptic conditions as strict as possible for these methods and dynamically, i.e., on Days 3, 8, 15 and 30. The collected samples were immediately placed in sterile plastic vials. All samples were frozen at −20 °C for subsequent DNA extraction.

To analyze the microbiome in the samples, DNA was isolated from the samples using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA) according to the manufacturer's instructions.

Amplification reactions for subsequent NGS sequencing were performed using a DTlite amplifier (DNA-Technology, LLC, Moscow, Russia) using eubacterial primers (Integrated DNA Technologies, Inc., USA): 343F (5'-CTCCTACGGRRSGCAGCAG-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3'), flanking the V1V3 region of the 16S rRNA gene. Metagenomic sequencing was performed using a MiSeq genomic sequencer (Illumina, Inc., USA) and the MiSeq Reagent Kit v3 (Illumina, Inc., USA). The maximum length of the obtained sequences was 2 × 300 bp. Chimeric sequences were excluded from the analysis using the USEARCH 7.0 program (http://drive5.com/usearch/). The obtained 2 × 300 bp reads were processed using the CLC Bio Genomics Workbench 7.0 platform for bioinformatics analysis (QIAGEN, The Netherlands) that included overlapping, quality filtering (QV > 15), and primer trimming. The taxonomic affiliation of microorganisms to genus was detected using the RDP Classifier program.

2.5. Statistical analysis

Mathematical and statistical processing of the results was performed using the multi-factor analysis of variance (ANOVA) method in Microsoft Excel XP/2003 and RStudio (Version 1.1.453; https://rstudio.com). The significance of differences was established using Student's t-test, and the differences were considered statistically significant at $p < 0.05$. In the case of metagenomic NGS data, statistical processing and plotting of diagrams were performed using Microsoft Excel XP/2003 and Past software; Pearson's coefficient was used to calculate correlations. The heatmap by families was constructed taking into account the representation at $p < 0.05$ (calculated in the limma package, in RStudio) using the moderated *t*-test.

3. Results and Discussion

3.1. Biochemical composition and nutritional value analysis

As a result of the biochemical analysis of the feeds produced in the model laboratory experiment, all the samples were characterized by high nutritional values (Table 2). The most important indicator of feed

is soluble crude protein, which indicates the availability of protein in the rumen of cattle. In our experiment, the highest crude protein value (17.01%) was found in sample No. 4 (n = 3). Another important indicator of feed for ruminants is fiber, which has a direct impact on the health of animals and their productivity, including milk quality. In our study, the most optimal NDF and ADF content was in silage samples Nos. 2 and 4. The highest ME values were recorded in samples Nos. 1 and 4. Sample No. 2 with two types of lactic acid bacteria in the starter had the highest lactic acid content. Lactic acid is the main preservative of silage, contributing to an increase in acidity and suppression of pathogenic, putrefactive microflora. Therefore, there is a strong correlation between lactic acid production and silage efficiency [25]. Herewith, it has good palatability, stimulating feed intake by cows.

Table 2 Analysis results of biochemical parameters of silage using bacterial strains

Indicator	**Sample**				
	Initial raw material	**1**	**2**	**3**	**4**
Mass fraction of dry matter, %	22.63[a]	22.60[a]	23.90[a]	21.30[a]	22.40[a]
Mass fraction of crude fat, %	3.23[a]	4.11[b]	3.93[b]	4.08[b]	4.11[b]
Mass fraction of crude protein, %	16.90[a]	16.90[a]	16.07[b]	16.34[b]	17.01[a]
Mass fraction of crude ash, %	9.3[a]	9.3[a]	11.3[b]	9.9[b]	10.3
Metabolizable energy, MJ/kg	9.07	8.98	8.70	8.92	8.97
Mass fraction of ammonia nitrogen, %	0.058[a]	0.265[b]	0.230[b]	0.290[b]	0.263[b]
Mass fraction of crude fiber, %	30.5	31.4	31.4	31.5	30.4
Acid detergent fiber, %	35.4	36.7	34.7	35.7	34.8
Neutral detergent fiber, %	64.6[a]	59.7 [b]	57.7 [b]	60.6[a]	57.1 [b]
Mass fraction of butyric acid, %	–	0.00	0.00	0.04	0.00
Mass fraction of lactic acid, %	–	1.92[a]	2.88[b]	1.64[c]	1.81[c]

Note: [a–c] values for the indicators conform to significant differences between samples at $p < 0.05$.

When analyzing the feeds, a rapid decline in pH in the feeds was demonstrated already on Day 2, which varied depending on the sample (Table 3). According to the data obtained, the lowest pH values (<4.4) on Day 30 were revealed for sample No. 2, i.e., 4.36. In general, the pH in sample No. 2, containing two bacterial strains, differed from the others in the maximum acidification level (pH = 4.28) achieved on Day 5 and stable maintenance of lower acidity throughout the experiment.

Table 3 Dynamics of pH level in samples using bacterial strains (n = 3)

Sample	pH						
	Day 1	Day 2	Day 3	Day 5	Day 8	Day 15	Day 30
Initial raw material	5.98						
1	4.71^{a}	4.47^{b}	4.36^{c}	4.30^{c}	4.34^{c}	4.38^{c}	4.37^{c}
2	4.69^{a}	4.49^{b}	4.45^{b}	4.28^{c}	4.38^{c}	4.34^{c}	4.36^{c}
3	4.71^{a}	4.61^{a}	4.61^{a}	4.57^{a}	4.72^{a}	4.58^{a}	4.58^{a}
4	4.66^{a}	4.47^{b}	4.42^{b}	4.34^{c}	4.50^{b}	4.50^{b}	4.46^{b}

Note: $^{a–c}$ values for the indicators conform to significant differences between samples depending on the treatment length at $p < 0.05$.

Correct choice and application of inoculants is paramount for good silage quality [26]. The use of homofermentative lactic acid bacteria strains, such as *L. plantarum*, can inhibit microbial growth in silage by accelerating the pH decline [27], while the heterofermentative strain *L. buchneri* can produce acetic acid to inhibit yeast and mold growth, thereby increasing the aerobic stability of silage.

Lactic acid bacteria can improve the aerobic stability of silage by producing various metabolites such as acetic acid, 1,2-propanediol, 3-phenyllactic acid and 3-hydroxydecanoic acid, as well as a phenolic antibiotic [28–30]. Studies have shown that many other metabolites are present in ensiled forage in addition to the common metabolites typically found when evaluating silage quality. In addition, they can produce a large number of metabolites during fermentation, such as amino acids, fatty acids, oligosaccharides, vitamins, small peptides, flavors and aroma compounds [31].

Studies by other authors confirm the positive effect of the combination of strains. Thus, our data are consistent with the study [32], in

which corn silage inoculated with co-cultures of *L. buchneri* and *L. plantarum* that together more effectively reduced pH and ammonia losses during fermentation compared to silages inoculated with only *L. buchneri*. Also, Ferrero et al. [33] tested a combination of two strains of obligate heterofermentative lactic acid bacteria *L. hilgardii* and *L. buchneri*. Treatment of silage using two strains showed a better result in increasing the aerobic stability of corn silage compared to treatment with only the *L. hilgardii* strain. These authors [33] suggested the existence of a synergistic effect between the two strains assessed and assumed that the increase in aerobic resistance was facilitated by the *L. hilgardii* strain that was dominant before *L. buchneri* became active.

3.2. Analysis of bacterial endotoxin genes

Next, to characterize the changes occurring in the silage under the influence of bacterial strains, the microbiota composition was examined using NGS sequencing in dynamics, i.e., on Day 1 (initial raw material) and Days 3, 8, 15 and 30 after the start of the experiment (Fig. 1). Bacteria of the phyla Firmicutes (52.56%) and Proteobacteria (31.85%) dominated in the initial plant mass. The subdominants were the phyla Bacteroidota (9.74%), Cyanobacteria (3.42%) and Actinobacteriota (1%) (Fig. 1). The dominant genus in the community was *Weissella* (48.35%), and the share of genera *Pseudomonas*, *Rhizobium*, *Leuconostoc*, *Stenotrophomonas*, *Serratia* and *Devosia* and the Erwiniaceae family ranged from 1 to 3%. The samples also contained Clostridium_sensu_stricto_9 (0.02%) and Enterobacteriaceae (2.09%) bacteria. On Day 3 of the experiment, the content of the Firmicutes phylum in the silage microbiome increased in all variants to 90–97%, including mainly three genera of lactobacilli: *Weissella*, *Lactobacillus* and *Pediococcus*. The major differences between the experimental samples were associated with the ratio of these genera. The highest proportion of the *Lactobacillus* genus was found in samples Nos. 1 (55.53%), 4 (51.32%) and 2 (45.34%).

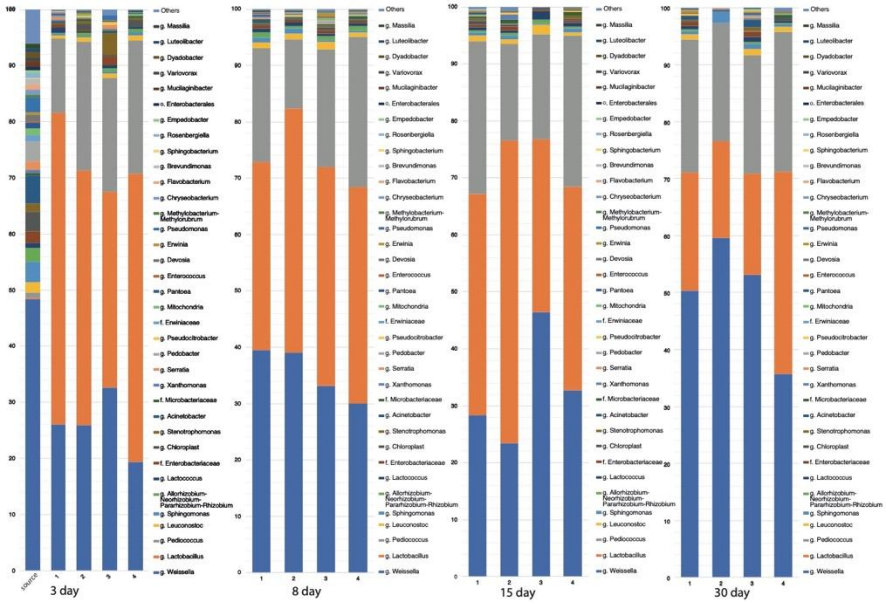


Fig. 1 Assessment results of the dynamics of the bacterial community at the genera level based on metagenomic NGS sequencing in ensiled feed in a model experiment ($n = 3$). Source, initial raw material (before the experiment); 1–4, samples Nos. 1, 2, 3 and 4

On Day 8, the dominance of lactobacilli of the genera *Weissella*, *Lactobacillus* and *Pediococcus* remained in all samples. On Day 15, the proportion of the genus *Lactobacillus* significantly decreased in sample No. 7 (by 1.3 times). In samples Nos. 1 and 2, there was an increase in bacteria of this genus by 1.2–1.5 times. At the end of the experiment, on Day 30, the proportion of *Lactobacillus* declined in all samples, except for sample No. 4. The content of the genus *Weissella* grew in all samples. The lowest content of this genus was in sample No. 4 (35.73%, $p < 0.05$), and the highest in sample No. 2 (59.2%). About 50–56% of *Weissella* were found in samples Nos. 1 and 3.

Overall, the relationship between fermentation time and microbial composition in our experiment was similar to the general succession pattern described by various authors and was crop-dependent. For example, in studies [27,34] with corn silage inoculated with either *L. plantarum* or *L. buchneri* and/or *L. hilgardii*, it could be observed that the succession of Firmicutes was rapid, i.e., within a few hours after sealing of the experimental mini-bins. A second observation was that members of the Leuconostocaceae family (mainly *Weissella* spp.)

were the dominant operational taxonomic unit during early fermentation.

An estimation of the correlations between the proportion of bacteria in silage and nutritional indicators (Table 4), for example, showed that the presence of bacteria of the genus *Lactobacillus* had a negative correlation with the butyric acid content in it −0.384), and those of the genus *Weissella* had a positive correlation with the crude fiber content (0.911). At the same time, both of these bacteria genera were a kind of antagonists and had a high level of negative correlation with each other (−0.968). Thus, our previous conclusion [35] that the high quality of silage is associated with the presence of bacteria of the genus *Lactobacillus* was confirmed here.

Table 4 Pearson correlation coefficients between biochemical components and quality indicators of ensiled bulk feeds and the presence of dominant microorganisms in them on Day 30 of the model experiment

	WEI	*LAC*	*PED*	DM	CFa	DP	DA	ME	CFi	BA	LA
WEI	1										
LAC	−0.968	1									
PED	−0.894	0.876	1								
DM	0.348	−0.130	−0.084	1							
CFa	−0.723	0.533	0.687	−0.746	1						
CP	−0.859	0.772	0.960	−0.304	0.853	1					
CA	0.311	−0.084	−0.411	0.669	−0.870	−0.648	1				
ME	0.911	0.527	0.702	−0.720	0.999	0.866	−0.881	1			
CFi	0.911	−0.983	−0.796	0.015	−0.375	−0.650	−0.099	−0.367	1		
BA	0.226	−0.384	−0.547	−0.782	0.174	−0.356	−0.238	0.140	0.418	1	
LA	0.599	−0.385	−0.427	0.933	−0.934	−0.626	0.804	−0.918	0.243	−0.506	1

Content indicators: *WEI*, *Weissella*; *LAC*, *Lactobacillus*; *PED*, *Pediococcus*; DM, dry matter; CFa, crude fat; CP, crude protein; CA, crude ash; ME, metabolizable energy; CFi, crude fiber; BA, butyric acid; LA, lactic acid. Green highlight: positive correlation coefficients; red highlight, negative correlation coefficients.

It can therefore be assumed that the quality of silage fermentation depends mainly on the microbial communities present and their succession, as well as the fermentative metabolites during ensiling. Understanding the processes underlying the production of quality silage

can provide a valuable scientific basis for improving the ensiling process and the quality of enhanced silage fermentation.

4. Conclusion

Ensiling of forages is extremely important where there are long and cold winters, high humidity, and where animals cannot obtain the energy or nutrients they need year-round from grazing. To improve the efficiency of enzymatic processes during ensiling, various lactic acid bacteria biopreparations are used, which can be divided into single-strain preparations and multi-strain preparations. Using microbiomic and bioinformation technologies and as a result of assessing the efficiency of using monocultures of lactic acid bacteria strains (*L. plantarum* 50 and *E. faecium* 46) and combining these strains for the ensiling process in a model laboratory experiment, it was shown that the greatest stability of the microbiome and a high proportion of lactobacilli, the best pH levels and silage quality were achieved when using a combination of *L. plantarum* 50 + *E. faecium* 46 strains. It is the most promising for a new preparation for harvesting bulk feed for cattle in the conditions of a risky farming zone This includes St. Petersburg and the Leningrad Oblast due to its geographical location and high humidity. The potential effect of its use is associated with ensuring the protection of animals from pathogenic bacteria (clostridia and enterobacteria) and their endotoxins, improving animal health and obtaining food products that are safe for human consumption.

Acknowledgments The present study was supported by the Russian Science Foundation (Grant No. 23-16-20007) and the St. Petersburg Science Foundation (Grant No. 23-16-20007, “Development of a comprehensive biotechnological approach for biological protection of cattle and livestock products from pathogenic bacteria and their toxins”).

References

1. Romanov, M.N., Bato, R.V., Yokoyama, M.T., Rust, S.R.: PCR detection and 16S rRNA sequence-based phylogeny of a novel *Propionibacterium acidipropionici* applicable for enhanced fermentation of high moisture corn. J. Appl. Microbiol. **97**, 38–47 (2004). https://doi.org/10.1111/j.1365-2672.2004.02282.x
2. Yang, J., Tan, H., Cai, Y.: Characteristics of lactic acid bacteria isolates and their effect on silage fermentation of fruit residues. J. Dairy Sci. **99**, 5325–5334 (2016). https://doi.org/10.3168/jds.2016-10952
3. Pahlow, G., Muck, R.E., Driehuis, F., Elferink, S.J.W.H.O.: Microbiology of ensiling. In: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.), Agronomy Monographs: Silage Science and Technology, Vol. 42, pp. 31–93. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI (2003). https://doi.org/10.2134/agronmonogr42.c2
4. Fabiszewska, A.U., Zielińska, K.J., Wróbel, B.: Trends in designing microbial silage quality by biotechnological methods using lactic acid bacteria inoculants: a minireview. World J. Microbiol. Biotechnol. **35**, 76 (2019). https://doi.org/10.1007/S11274-019-2649-2
5. McAllister, T.A., Dunière, L., Drouin, P., Xu, S., Wang, Y., Munns, K., Zaheer, R.: *Silage review*: Using molecular approaches to define the microbial ecology of silage. J. Dairy Sci. **101**, 4060–4074 (2018). https://doi.org/10.3168/jds.2017-13704
6. Li, D.X., Ni, K.K., Zhang, Y.C., Lin, Y.L., Yang, F.Y.: Influence of lactic acid bacteria, cellulase, cellulase-producing *Bacillus pumilus* and their combinations on alfalfa silage quality. J. Integr. Agric. **17**, 2768–2782 (2018). https://doi.org/10.1016/S2095-3119(18)62060-X
7. Ben-Dov, E., Shapiro, O.H., Siboni, N., Kushmaro, A.: Advantage of using inosine at the 3′ termini of 16S rRNA gene universal primers for the study of microbial diversity. Appl. Environ. Microbiol. **72**, 6902–6906 (2006). https://doi.org/10.1128/AEM.00849-06
8. Gaucher, F., Bonnassie, S., Rabah, H., Leverrier, P., Pottier, S., Jardin, J., Briard-Bion, V., Marchand, P., Jeantet, R., Blanc, P.,

Jan, G.: Benefits and drawbacks of osmotic adjustment in *Propionibacterium freudenreichii*. J. Proteom. **204**, 103400 (2019). https://doi.org/10.1016/j.jprot.2019.103400
9. Santos, A.O., Ávila, C.L.S., Schwan, R.F.: Selection of tropical lactic acid bacteria for enhancing the quality of maize silage. J. Dairy Sci. **96**, 7777–7789 (2013). https://doi.org/10.3168/jds.2013-6782
10. Wang, X., Haruta, S., Wang, P., Ishii, M., Igarashi, Y., Cui, Z.: Diversity of a stable enrichment culture which is useful for silage inoculant and its succession in alfalfa silage. FEMS Microbiol. Ecol. **57**, 106–115 (2006). https://doi.org/10.1111/j.1574-6941.2006.00099.x
11. Ren, H., Wang, L., Sun, Y., Zhao, Q., Sun, Y., Li, J., Zhang, B.: Enhancing the co-ensiling performance of corn stover and cabbage waste via the addition of cellulase. Bioresources **16**, 6342–6362 (2021). https://doi.org/10.15376/biores.16.3.6342-6362
12. Pretz, J.: Understanding the process of corn silage fermentation and starch availability. Hubbard Feeds, Mankato, MN, USA (2020). https://www.hubbardfeeds.com/blog/understanding-process-corn-silage-fermentation-and-starch-availability. Accessed 6 Mar 2025.
13. Liu, Q.H., Yang, F.Y., Zhang, J.G., Shao, T.: Characteristics of *Lactobacillus parafarraginis* ZH1 and its role in improving the aerobic stability of silages. J. Appl. Microbiol. **117**, 405–416 (2014). https://doi.org/10.1111/jam.12530
14. Li, Y., Nishino, N.: Monitoring the bacterial community of maize silage stored in a bunker silo inoculated with *Enterococcus faecium*, *Lactobacillus plantarum* and *Lactobacillus buchneri*. J. Appl. Microbiol. **110**, 1561–1570 (2011). https://doi.org/10.1111/j.1365-2672.2011.05010.x
15. Bai, J., Ding, Z., Ke, W., Xu, D., Wang, M., Huang, W., Zhang, Y., Liu, F., Guo, X.: Different lactic acid bacteria and their combinations regulated the fermentation process of ensiled alfalfa: ensiling characteristics, dynamics of bacterial community and their functional shifts. Microb. Biotechnol. **14**, 1171 (2021). https://doi.org/10.1111/1751-7915.13785
16. Kim, D., Lee, K.D., Choi, K.C.: Role of LAB in silage fermentation: Effect on nutritional quality and organic acid production—

An overview. AIMS Agric. Food **6**, 216–234 (2021). https://doi.org/10.3934/AGRFOOD.2021014

17. Zhao, S., Yang, F., Wang, Y., Fan, X., Feng, C., Wang, Y.: Dynamics of fermentation parameters and bacterial community in high-moisture alfalfa silage with or without lactic acid bacteria. Microorganisms **9**, 1225 (2021). https://doi.org/10.3390/MICROORGANISMS9061225
18. Tian, J., Li, Z., Yu, Z., Zhang, Q., Li, X.: Interactive effect of inoculant and dried jujube powder on the fermentation quality and nitrogen fraction of alfalfa silage. Anim. Sci. J. **88**, 633–642 (2017). https://doi.org/10.1111/asj.12689
19. Muck, R.E., Nadeau, E.M.G., McAllister, T.A., Contreras-Govea, F.E., Santos, M.C., Kung Jr, L.: Silage review: Recent advances and future uses of silage additives. J. Dairy Sci. **101**, 3980–4000 (2018). https://doi.org/10.3168/JDS.2017-13839
20. Nazar, M., Wang, S., Zhao, J., Dong, Z., Li, J., Kaka, N.A., Shao, T.: The feasibility and effects of exogenous epiphytic microbiota on the fermentation quality and microbial community dynamics of whole crop corn. Bioresour. Technol. **306**, 123106 (2020). https://doi.org/10.1016/j.biortech.2020.123106
21. Laptev, G.Yu., Yildirim, E.A., Ilina, L.A., Ponomareva, E.S., Kalitkina, K.A., Turina, D.G., Filippova, V.A., Dubrovin, A.V., Bashir, Kh., Smetannikova, T.S., Malakhov, I.G., Novikova, N.I., Romanov, M.N.: Effect of a probiotic strain administration in different feeding phases on α- and β-diversity and gene expression of the rumen microbiome in lactating cows. In: Ronzhin, A., Kostyaev, A. (Eds.), Smart Innovation, Systems and Technologies: Proceedings of the Third International Conference on Agriculture Digitalization and Organic Production (ADOP 2023), Vol. 362, pp. 181–191. Springer Nature Singapore Pte Ltd., Singapore (2023). https://doi.org/10.1007/978-981-99-4165-0_17
22. Yildirim, E., Ilina, L., Laptev, G., Tyurina, D., Filippova, V., Dubrovin, A., Novikova, N., Kalitkina, K., Djepbarova, O., Ponomareva, E., Dubrovina, A., Klyuchnikova, I., Patyukova, N., Griffin, D., Romanov, M.: The search for sources of enterobacteria and clostridia endotoxins in Russian dairy farms: possible transfer of endotoxins through the feed-cow-milk chain. In: Ronzhin, A., Bakach, M., Kostyaev, A. (Eds.), Smart Innovation, Systems and Technologies: Proceedings of the Fourth International Conference

on Agriculture Digitalization and Organic Production (ADOP 2024), Vol. 397, pp. 325–335. Springer Nature Singapore Pte Ltd., Singapore (2024). https://doi.org/10.1007/978-981-97-4410-7_27

23. Yildirim, E.A., Laptev, G.Y., Ilina, L.A., Ponomareva, E.S., Brazhnik, E.A., Smetannikova, T.S., Novikova, N.I., Tyurina, D.G., Filippova, V.A., Dubrovin, A.V., Dubrovina, A.S., Kalitkina, K.A., Klyuchnikova, I.A., Zaikin, V.A., Griffin, D.K., Romanov, M.N.: Metagenomic composition and predicted metabolic pathway analyses of the endometrial and rectal microbiota in dairy cows following the introduction of a complex feed additive. Front. Biosci. (Elite Ed.) **17**, 25725 (2025). https://doi.org/10.31083/FBE25725
24. Bondarev, V.A., Kosolapov, V.M., Pobednov, Yu.A., Panov, A.A., Akhlamov, Yu.D., Otroshko, S.A., Popov, V.V., Sokolkov, V.M., Klimenko, V.P., Mamaev, A.A.: [Conducting Experiments on Preservation and Storage of Bulk Feed (methodological recommendations)]. Federal State Institution RCSC, Moscow, Russia (2008). https://www.vniikormov.ru/pdf/provedenie-opytov-po-konservirovaniyu-i-hraneniyu-obemistyh-kormov.pdf. Accessed 6 Mar 2025.
25. Zhao, X., Sun, Y., Chang, Z., Yao, B., Han, Z., Wang, T., Shang, N., Wang, R.: Innovative lactic acid production techniques driving advances in silage fermentation. Fermentation **10**, 533 (2024). https://doi.org/10.3390/fermentation10100533
26. Drouin, P., Mari, L.J., Schmidt, R.J.: Lactic acid bacteria as microbial silage additives: Current status and future outlook. In: Martínez-Espinosa, R.M. (Ed.), New Advances on Fermentation Processes, pp. 153–176. IntechOpen, London, UK (2020). https://doi.org/10.5772/intechopen.89326
27. Keshri, J., Chen, Y., Pinto, R., Kroupitski, Y., Weinberg, Z.G., Sela, S.: Microbiome dynamics during ensiling of corn with and without *Lactobacillus plantarum* inoculant. Appl. Microbiol. Biotechnol. **102**, 4025–4037 (2018). https://doi.org/10.1007/s00253-018-8903-y
28. Reich, L.J., Kung Jr, L.: Effects of combining *Lactobacillus buchneri* 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. Anim. Feed Sci. Technol. **159**, 105–109 (2010). https://doi.org/10.1016/j.anifeedsci.2010.06.002

29. Broberg, A., Jacobsson, K., Ström, K., Schnürer, J.: Metabolite profiles of lactic acid bacteria in grass silage. Appl. Environ. Microbiol. **73**, 5547–5552 (2007). https://doi.org/10.1128/AEM.02939-06
30. Valan Arasu, M., Jung, M.W., Ilavenil, S., Jane, M., Kim, D.H., Lee, K.D., Park, H.S., Hur, T.Y., Choi, G.J., Lim, Y.C., Al-Dhabi, N.A., Choi, K.C.: Isolation and characterization of antifungal compound from *Lactobacillus plantarum* KCC-10 from forage silage with potential beneficial properties. J. Appl. Microbiol. **115**, 1172–1185 (2013). https://doi.org/10.1111/jam.12319
31. Sun, M.C., Li, A.L., Huo, G.C., Meng, X.C.: Progress on the metabolomics of lactic acid bacteria. Microbiology Weishengwuxue Tongbao **39**, 1499–1505 (2012). https://qikan.cqvip.com/Qikan/Article/Detail?id=43571176. Accessed 6 Mar 2025.
32. Filya, I.: The effect of *Lactobacillus buchneri*, with or without homofermentative lactic acid bacteria, on the fermentation, aerobic stability and ruminal degradability of wheat, sorghum and maize silages. J. Appl. Microbiol. **95**, 1080–1086 (2003). https://doi.org/10.1046/j.1365-2672.2003.02081.x
33. Ferrero, F., Piano, S., Tabacco, E., Borreani, G.: Effects of conservation period and Lactobacillus hilgardii inoculum on the fermentation profile and aerobic stability of whole corn and sorghum silages. J. Sci. Food Agric. **99**, 2530–2540 (2018). https://doi.org/10.1002/jsfa.9463
34. Gerlach, K., Südekum, K.H.: How do time of fermentation and lactic acid bacteria inoculation influence microbial succession during ensiling? In Advances in Silage Research: XVIII International Silage Conference, pp. 32–33. University of Bonn, Bonn, Germany (2018). https://www.cabidigitallibrary.org/doi/pdf/10.5555/20193168094. Accessed 6 Mar 2025.
35. Filippova, V., Yildirim, E., Ilina, L., Laptev, G., Dubrovin, A., Zaikin, V., Kalitkina, K., Dubrovina, A., Malakhov, I., Klyuchnikova, I.: Analysis of the presence of toxins from Escherichia coli, Clostridium perfringens, C. difficile in bulk feeds of Saint-Petersburg and Leningrad Region. Sib. J. Life Sci. Agric. **16**, 164–185 (2024). https://doi.org/10.12731/2658-6649-2024-16-4-858