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Effects of *Lacticaseibacillus paracasei and Enterococcus faecium* on the microbial community and metabolic potential of legume-cereal silage

Sophia J. Gallegos[1] ✉, **Ekaterina S. Ponomareva**[1], **Larisa A. Ilina**[2]

[1]ITMO University, *Saint Petersburg, Russian Federation*
[2]Saint Petersburg State Agrarian University, *Pushkin, Russian Federation*
✉ gallegos_sophia@outlook.com

Abstract. Silage is used as a major part of fodder all over the world. Lactic acid bacteria (LAB) play a critical role in silage production from legumes, grains, and other plants, lowering the pH by producing different kinds of organic acids during ensiling, which inhibits undesirable bacteria that decrease the quality and shelf life of the inoculated product. The aim of this study was to determine the effects of *Lacticaseibacillus paracasei* and *Enterococcus faecium* inoculated together and separately on the bacterial community, fermentation quality and metabolic pathways of legume-cereal silage after 30 days. We conducted a model ensiling of legume-cereal crops of the first harvest cut in the Leningrad region at the booting stage. Three experimental groups with different bacterial strains were added during ensiling (LE: *L. paracasei* and *E. faecium*; LP: *L. paracasei*; and EF: *E. faecium*). The microorganisms in the silage were counted at 0 and 30 days of ensiling, using Illumina MiSeq high-throughput sequencing to determine their composition. The DNA of the silage microbial community was isolated, amplified, sequenced, and processed, then used to predict functional metabolic pathway abundances based on 16S rRNA marker gene sequences using the database MetaCyc. LAB genera were presented as minor taxa pre-ensiling, while *Weissella*, *Lactobacillus*, and *Pediococcus* were the dominant genera after 30 days of silage. Overall, LE had the most desirable bacteria and metabolic pathways for silage. LP also performed

favorably. EF had undesirable bacteria and metabolites, showing the worst fermentation quality. Our results found important differences in predicted carbohydrate and biogenic amine metabolism between the different groups as well. To our knowledge, little information is available regarding the dynamics of bacterial community succession and metabolic pathways of silage prepared with *L. paracasei* and *E. faecium*.

Key words: ensilage, lactic acid bacteria, bacterial community, metabolic pathways

Authors' contribution: Gallegos S.J. — statistical analysis and interpretation of results, preparation of the manuscript, editing; Ponomareva E.S. — carrying out molecular genetic analysis and general scientific supervision; Ilina L.A. — planning and organizing the experiments, selecting research materials, approval of the final manuscript version. All authors approved the final version of the manuscript.

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Introduction

In many parts of the world preserved forages, such as silages, are used as a major part of the diet of livestock. Since ensiling is a consistent and reliable method for producing feed, it is becoming increasingly popular. Lactic acid bacteria (LAB) play a critical role in silage production from legumes, grains, and other plants. In anaerobic conditions, LAB lower the pH during ensiling, which inhibits undesirable bacteria and increases the shelf life of the inoculated product [1].

Legume crops are associated with more diverse microbial populations and higher protein than cereal crops. Higher protein results in the growth of undesirable microbes and deterioration of fermentation quality. Ensiling legume crops with cereal crops improves silage quality by decreasing the pH and increasing the content of lactic and acetic acid [2, 3].

The fermentation quality of silage is highly dependent on the succession of the microbial community during ensiling. The predomination or survival of undesirable microorganisms results in poor fermentation quality; silage loses energy and nutrients [4].

Since different LAB species have different fermentation characteristics, single LAB species often cannot achieve optimal fermentation results. Some inoculant preparations contain synergistic mixtures of bacteria that target different phases of the fermentation. For example, many combination inoculants contain *E. faecium*, with grows rapidly at high pH (>5), dominating early stages of the fermentation phase, before other LAB strains take over once the pH drops below 5 [5]. Composite inoculants work by combining multiple LAB species that enhance each other's growth and function, leading to better silage fermentation [6].

Since silage fermentation involves intricate microbial communities and metabolic pathways during ensiling, different inoculants produce distinct variations in both microbial composition and metabolic profiles. Multiple microbial and metabolic dynamics are correlated and affect the fermentation process [7, 8].

To our knowledge, little information is available regarding the dynamics of microbial community succession and predicted functional metabolic pathways of silage prepared with facultative heterofermenters *L. paracasei* and *E. faecium* [5, 9]. A better understanding of microbial metabolic pathways underlying silage fermentation could provide us with important information to regulate silage fermentation [10].

The objective of this study was to analyze the change in the diversity of silage microbial community during the ensiling process, evaluate the efficacy of two microbial strains for use as inoculants and further understand the dynamics of metabolic pathways of the microbial community that occur during ensiling.

Methods and materials

We conducted a model ensiling of legume-cereal crops of the first harvest cut in the Leningrad region at the booting stage at a moisture content of 84.7% ± 1.3. The experiment lasted 30 days and was conducted in the BIOTROF LLC laboratory. All inoculations were applied using a manual sprayer after green forage was weighed and uniformly distributed on polyethylene film. After, the forage was compacted and sealed in 2 kg vacuum bags, then subsequently stored under temperature conditions approximating real-world conditions. Three experimental groups with different bacterial strains were added during ensiling. The first group had *L. paracasei* mixed with *E. faecium* (LE), the second had just *L. paracasei* (LP), and the third had *E. faecium* (EF).

The microorganisms in the silage were counted at 0 and 30 days of ensiling. PCR analysis was performed in the same laboratory. Illumina MiSeq high-throughput sequencing was used to determine the composition of microorganisms. DNA isolation of the silage microbial community was performed using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. A Verity DNA Amplifier (Life Technologies, Inc., USA) was used for amplification for subsequent NGS sequencing using eubacterial primers (IDT), 343F (5'- CTCCTACTACGGRRSGCAGCAG-3') and 806R (5'-GGACTACNVGGGTWTCTAAT3'), flanking the V1V3 region of the 16S rRNA gene. Metagenomic sequencing was performed on a MiSeq genomic sequencer (Illumina, Inc., USA) with the MiSeq Reagent Kit v3 (Illumina, Inc., USA). The maximum length of the sequences obtained was 2 × 300 nt. Chimeric sequences were excluded from analysis using the program "USEARCH 7.0" (http://drive5.com/usearch/). The obtained 2 × 300 nt reads were processed using the software "CLC Bio GW 7.0" (Qiagen, The Netherlands) and included overlapping, quality filtering (QV > 15), and primer trimming. Taxonomic affiliation of microorganisms to the genus was determined using the RDP Classifier program. Statistical processing of data and construction of

diagrams were carried out using Microsoft Excel, Phantasus (https://artyomovlab.wustl.edu/phantasus/), and Datawrapper (https://www.datawrapper.de/). PICRUSt2 (v.2.3.0) (https://github.com/picrust/picrust2) was used for a comparative analysis of the presence of specific bacterial gene families, predicting functional abundances based on 16S rRNA marker gene sequences using the database MetaCyc [11].

Results and discussion

On day 0, *Proteobacteria*, *Bacteroidota* and *Firmicutes* were the dominant phyla, with a total of 94.08% bacteria classified into these phyla. As the ensiling progressed, microbial diversity significantly decreased: on day 0 taxonomic analysis indicated that the bacterial community was classified into 12 phyla and 134 distinct genera, while by the end of fermentation LE had 6 phyla and 36 distinct genera; LP had 6 phyla and 45 distinct genera; and EF had 8 phyla and 124 distinct genera, indicating that great changes had taken place after 30 days of fermentation.

After 30 days, the dominant phyla shifted from *Proteobacteria* to *Firmicutes* for all 3 experimental groups (Fig. 1). *Firmicutes* increased from 23.23% on day 0 to 96.79% for LE, 96.57% for LP, and 94.95% for EF, while *Proteobacteria* and *Bacteroidota* both greatly decreased for LE, LP and EF (from 58.52 to 2.03%, 1.65 and 1.51% and 12.33 to 0.05%, 0.22 and 2.08% respectively).

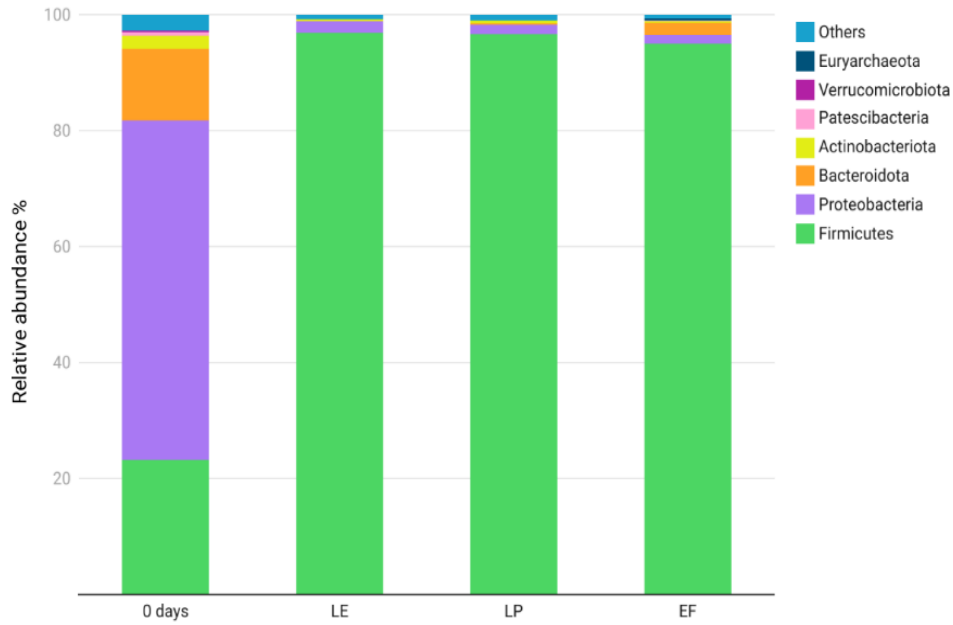


Fig. 1. Bacterial communities and relative abundance by phylum level for legume-cereal silage after 30 days ensiling: LE – silage inoculated with *L. paracasei* and *E. faecium*; LP – silage inoculated with *L. paracasei*; EF – silage inoculated with *E. faecium*

Source: compiled by S.J. Gallegos with Datawrapper.

By the end of 30 days, variants LE and LP had similar microbial communities, with only slight differences in number, while EF differed. Overall, LAB dominated; with families *Lactobacillaceae* and *Leuconostocaceae* flourishing. Generally, the main LAB genera that play a major role in silages fermentation include *Weissella*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Enterococcus* and *Pediococcus* [12, 13]. Our study supported those findings, as the dominant bacteria found after ensiling were *Weissella*, *Lactobacillus*, *Pediococcus*, and *Leuconostoc*, in that order, as shown in Fig., 2*a*.

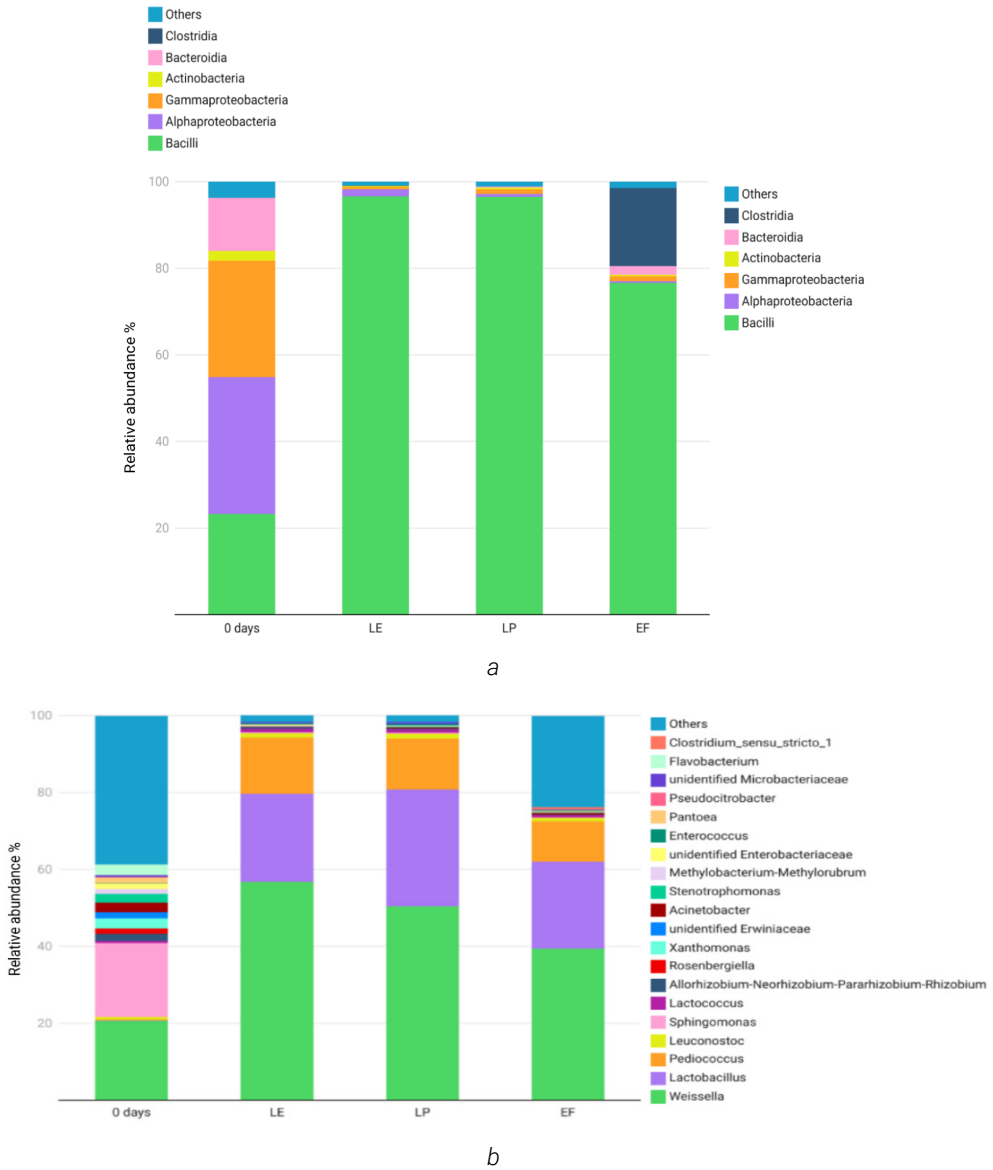


Fig. 2. Bacterial communities and relative abundance by class level (*a*) and genus level (*b*) for legume-cereal silage after 30 days ensiling: LE — silage inoculated with *L. paracasei* and *E. faecium*; LP — silage inoculated with *L. paracasei*; EF — silage inoculated with *E. faecium*

Source: compiled by S.J. Gallegos with Datawrapper.

Weissella dominated and more than doubled in LE and LP: increasing from 20.74% to 56.81 and 50.44% respectively, while EF overall only had a slight increase to 39.38%. *Lactobacillus* rose from 0.10% to 22.88, 30.31 and 22.71% and *Pediococcus* rose from 0.16% to 14.61, 13.26 and 10.46%.

For the non-LAB — the genus *Sphingomonas* rapidly decreased in number in all variants: from 19.27% on day 0 to 0.17, 0.26 and 0.11% on day 30 respectively, as shown in Fig., 2*b*. *Sphingomonas* is found on fresh forages and acts as a harmful component in silage, increasing pH level and degrading protein. *Sphingomonas* is negatively correlated with the fermentation acids of the silages, such as lactic and acetic acids, and consequently the whole fermentation process [14].

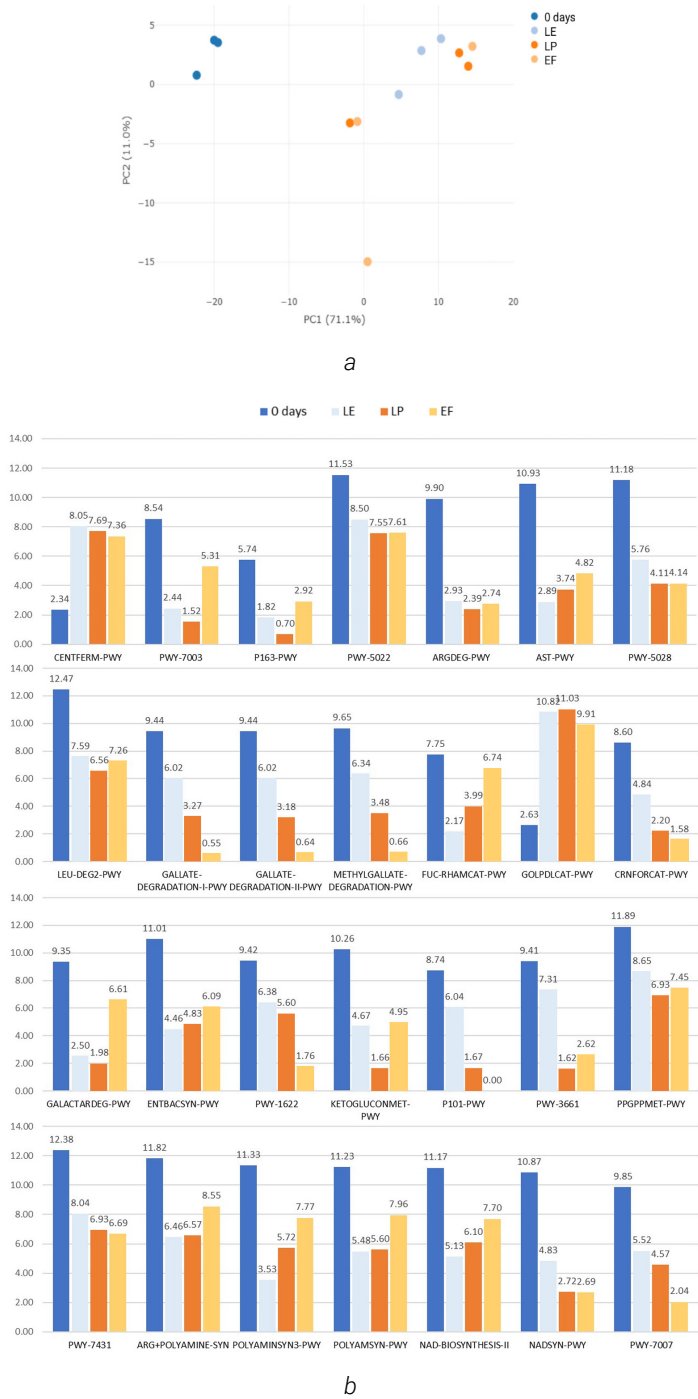
The number of *Clostridia* rapidly rose in the EF variant. EF had 18.02% *Clostridia* by day 30, compared to 0.08% in LE and 0.01% in LP. Silage quality is degraded by the presence of *Clostridium spp.* and when consumed, such silage jeopardizes animal health and productivity. *Clostridium* in silage converts sugars and proteins to butyric acid [15]. Butyric acid is associated with poorly fermented silage, being accompanied by a range of undesirable by-products, including amines, ammonia, and acetic acid [16]. High amounts of *Clostridia* in the EF group indicate that the silage underwent clostridial fermentation, has high amounts of butyric acid and is low in nutritional value.

In total 400 metabolic pathways were predicted. Out of those only 263 were statistically significant ($p < 0.05$). Differences in various metabolic pathways with specific biofunctions were found among the inoculant groups. By day 30, LE had the least variation among statistically significant metabolic pathways, while EF had the most variation, as shown in Fig., 3*a*.

Multiple metabolic pathways were predicted that are associated with butanoate: CENTFERM-PWY, PWY-5022, PWY-7003, and P163-PWY. EF had the highest levels for both PWY-7003 (glycerol degradation to butanol) and P163-PWY (L-lysine fermentation to acetate and butanoate); both of which are associated with the production of undesirable butanoate and ammonia.

The metabolic pathway of enterobactin biosynthesis (ENTBACSYN-PWY) was also predicted to have highest levels in EF (Fig., 3*b*). Enterobactin is produced almost exclusively by *Enterobacteria*, which are detrimental to have in silage as they compete with lactic acid bacteria for the available sugars, reduce the nutritional value of silage and break down nitrates in the ensiled material, forming ammonia and making it more difficult to quickly lower the pH of the silage [17].

Several metabolic pathways involved with biosynthesizing polyamines were predicted (ARG+POLYAMINE-SYN, POLYAMINSYN3-PWY and POLYAMSYN-PWY). All three were found the least in LE and the most in EF. Biogenic amines, like putrescine and tyramine, are a group of positively charged nitrogenous compounds which are considered detrimental to silage. Biogenic amines are found to jeopardize the health of those who consume them and may exert detrimental impacts on feed intake and preference of ruminants [18, 19]. The activities of some undesirable bacterial genera (*Clostridia*, *Pseudomonas*, *Enterobacteria*) and slow acidification result in the accumulation of biogenic amines during ensiling [20].



a

b

Fig. 3. Statistically significant ($p < 0.05$) 16S rRNA gene-predicted metabolic pathways: ***a*** — principal coordinates analysis (PCoA) for statistically significant metabolic pathways of legume-cereal silage after 30 days of ensiling; ***b*** — bar graphs showing 28 chosen statistically significant metabolic pathways on day 0 and day 30 for LE, LP, and EF; LE — silage inoculated with ***L. paracasei*** and ***E. faecium***; LP — silage inoculated with ***L. paracasei***; EF — silage inoculated with ***E. faecium***

Source: compiled by S.J. Gallegos with Phantasus (***a***) and Microsoft Excel (***b***).

Correspondingly, the desirable aromatic biogenic amine degradation metabolic pathway used by bacteria (PWY-7431) was predicted to be highest for LE and lowest for EF. However, the predicted superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation (ARGDEG-PWY) had similar levels for all three groups.

Two metabolic pathways associated with osmotic stress corresponded with each other. The glycine betaine degradation pathway (PWY-3661) and ectoine biosynthesis metabolic pathway (P101-PWY) were predicted by far the most in LE, with comparatively very low amounts on LP and EF. Glycine betaine acts as an osmoprotectant, accumulating in high cytoplasmic concentrations in response to osmotic stress, as does ectoine, which helps maintain membrane stability during fermentation [21]. This supports the idea that ectoine was used by the bacteria in LE to protect themselves from osmotic stress during fermentation, while the other two groups used glycine betain.

The metabolic superpathway of fucose and rhamnose degradation (FUC-RHAMCAT-PWY) yields glycerone phosphate, which directly enters central metabolism, and lactaldehyde, which is reduced to 1,2-propanediol under anaerobic conditions and then secreted into the environment. EF was predicted to have similarly high levels of this pathway as day 0, while LE and LP had much lower levels. 1,2-propanediol is secreted by lactic acid bacteria and has antimicrobial activity. In addition, 1,2-propanediol is good for silage aerobic stability, is often fed to ruminants to prevent ketosis, and plays an important role in livestock health [2, 22].

For all three predicted gallate degradation metabolic pathways (GALLATE-DEGRADATION-I-PWY, GALLATE-DEGRADATION-II-PWY and METHYLGALLATE-DEGRADATION-PWY) LE had the highest levels while EF had practically none. All three pathways directly contribute to generating energy and creating pyruvate, which bacteria use to metabolize into lactate, acetate, formate, and other metabolites. In addition, other studies found that a higher abundance of pyruvate metabolism, among a few other pathways, was suggested as a possible reason for enhancing silage stability [23, 24].

Conclusion

The quality of silage depends heavily on achieving rapid acidification through LAB while preventing the growth of undesirable microorganisms like *Clostridia* and *Enterobacteria*. Used together as a combination inoculant, *L. paracasei* and *E. faecium* created more favorable conditions for the development of LAB and, consequently, produced high-quality silage. Comparatively, *E. faecium* by itself created the least favorable conditions: with 88 more distinct genera, 225-fold more *Clostridia*, and 50% more predicted polyamine biosynthesizing metabolic pathways. As a result of this study, we learned about the metabolic pathways involved during ensilage with *L. paracasei* and *E. faecium*, which can provide insight into microbial, metabolic, and silage parameters; as well as be used as potential biomarkers for further research.

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Влияние *Lacticaseibacillus paracasei и Enterococcus faecium* на микробное сообщество и метаболический потенциал силоса бобово-злаковых культур

С.Д. Галлегос[1] ✉, Е.С. Пономарева[1], Л.А. Ильина[2]

[1]Национальный исследовательский университет ИТМО, *г. Санкт-Петербург, Российская Федерация*

[2]Санкт-Петербургский государственный аграрный университет, *г. Пушкин, Российская Федерация*

✉ gallegos_sophia@outlook.com

Аннотация. Силос используется в качестве основного кормового компонента во всем мире. Молочнокислые бактерии (МЛБ) играют ключевую роль в производстве силоса из бобовых, зерновых и других растений, снижая pH за счет продукции различных органических кислот во время силосования. Это подавляет рост нежелательных бактерий, которые ухудшают качество и срок хранения инокулированного продукта. Целью исследования являлось определение влияния раздельного и совместного добавления *Lacticaseibacillus paracasei* и *Enterococcus faecium* на состав бактериального сообщества и метаболические пути силоса бобово-злаковых культур после 30 дней ферментации. Было проведено модельное силосование бобово-злаковых культур первого укоса в Ленинградской области на фазе выхода в трубку. В процессе силосования были сформированы три экспериментальные группы с разными бактериальными штаммами: LE (*L. paracasei* + *E. faecium*), LP (*L. paracasei*) и EF (*E. faecium*). Микроорганизмы в силосе анализировали на 0 и 30 день силосования с помощью высокопроизводительного секвенирования на платформе Illumina MiSeq. ДНК микробного сообщества выделяли, амплифицировали, секвенировали и обрабатывали с последующим прогнозированием обилия функциональных метаболических путей на основе последовательностей маркерного гена 16S рРНК с использованием базы данных MetaCyc. До силосования роды МЛБ были представлены в меньшинстве, однако после 30 дней ферментации доминирующее положение заняли *Weissella*, *Lactobacillus* и *Pediococcus*. Группа LE продемонстрировала наиболее желательный состав бактерий и метаболических путей. Группа LP также показала хорошие результаты. Группа EF

характеризовалась наличием нежелательных бактерий и метаболитов, что указывает на наихудшее качество брожения. Полученные результаты выявили значимые различия в прогнозируемом метаболизме углеводов и биогенных аминов между экспериментальными группами. Насколько нам известно, данные о динамике сукцессии бактериального сообщества и метаболических путей силоса, приготовленного с использованием *L. paracasei* и *E. faecium*, представлены в литературе крайне ограниченно.

Ключевые слова: силосование, молочнокислые бактерии, бактериальное сообщество, метаболические пути

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About the authors:
Gallegos Sophia Julia — Bachelor's student, Faculty of Biotechnology, ITMO University, 49A Kronverksky ave., Saint Petersburg, 197101, Russian Federation; e-mail: gallegos_sophia@outlook.com
ORCID: 0009-0004-9981-3016
Ponomareva Ekaterina Sergeevna — Engineer, Advanced Engineering School of Interdisciplinary Engineering, ITMO University, 49A Kronverksky ave., Saint Petersburg, 197101, Russian Federation; e-mail: olololven@mail.ru
ORCID: 0000-0002-4336-8273 SPIN-code: 4260-6755
Ilyina Larisa Aleksandrovna — Doctor of Biological Sciences, Professor, Department of Large Livestock Farming, Saint Petersburg State Agrarian University; 2A Peterburgskoe highway, Pushkin, Saint Petersburg, 196601, Russian Federation; e-mail: ilina@biotrof.ru
ORCID: 0000-0003-2789-4844 SPIN-code: 5826-7525

Об авторах:
Галлегос София Джулия — студент бакалавриата факультета биотехнологии, Национальный исследовательский университет ИТМО, Российская Федерация, 197101, г. Санкт-Петербург, Кронверкский проспект, д. 49, лит. А; e-mail: gallegos_sophia@outlook.com
ORCID: 0009-0004-9981-3016
Пономарева Екатерина Сергеевна — инженер Передовой инженерной школы интердисциплинарного инжиниринга, Национальный исследовательский университет ИТМО, Российская Федерация, 197101, г. Санкт-Петербург, Кронверкский проспект, д. 49, лит. А; e-mail: olololven@mail.ru
ORCID: 0000-0002-4336-8273 SPIN-код: 4260-6755
Ильина Лариса Александровна — доктор биологических наук, профессор кафедры крупного животноводства, Санкт-Петербургский государственный аграрный университет, Российская Федерация, 196601, г. Санкт-Петербург, г. Пушкин, Петербургское шоссе, д. 2, лит. А; e-mail: ilina@biotrof.ru
ORCID: 0000-0003-2789-4844 SPIN-код: 5826-7525