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THE INVESTIGATION OF ENDOPHYTIC MICROORGANISMS AS A SOURCE FOR SILAGE MICROBIOCENOSIS FORMATION USING NGS-SEQUENCING

E.A. YILDIRIM[1], G.Yu. LAPTEV[1], L.A. IL'INA[1], I.N. NIKONOV[1], V.A. FILIPPOVA[1], V.V. SOLDATOVA[1], E.A. BRAZHNIK[1], N.I. NOVIKOVA[1], T.Yu. GAGKAEVA[2]

[1]*Biotrof+ Ltd,* 7-N, 8, lit. A, Malinovskaya ul., St. Petersburg, 196602 Russia, e-mail deniz@biotrof.ru, laptev@biotrof.ru, ilina@biotrof.ru, nikonov@biotrof.ru, dumova@biotrof.ru, biotrof@biotrof.ru, bea@biotrof.ru;
[2]*All-Russian Research Institute of Plant Protection,* Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail t.gagkaeva@mail.ru

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Abstract

The composition of plant and silage microflora affects the fermentation processes in the silage and its final quality. To date, reports about studying fodder plants and silage microbiota by means of molecular genetic methods are few and limited to descriptions of composition and function of some groups of microorganisms. Moreover, the NGS (next generation sequencing) data on diversity of epiphytic microflora and silage microbiocoenosis are not still reported. We first used this approach in studying phyllosphere and silage microbiom, and reported it to be rather rich in composition and abundance that is in contrast with conventional understanding. At that, the pathogenic and non-culturable microbes were detected in the microbiota, including specific inhabitants of mammalian gastrointestinal tract. So using NGS we examined the structure and diversity of bacterial community of *Dactylis glomerata* L. harvested plants and the biomass ensilaged with chemical preservative AIV 2000 Plus (KEMIRA OYJ Inc., Finland) composed of mixture of formic, propionic and benzoic acids. Assays were carried out on days 3, 7, 14 and 30 of ensilaging. The results showed that the bacterial community of silage from *D. glomerata* sharply differed from the composition of foliage microorganisms and varied greatly in the course of successive changes which occurred during maturation of silage preserved by mixture of organic acids. The composition of plant microorganisms and silage were found to be very various in contrast with a traditional view. Among foliar microorganisms of *D. glomerata* there were mostly the bacteria of phylum *Proteobacteria* (94.1 %), and in the silage the bacteria of phylums *Bacteroidetes* and *Firmicutes* were main representatives (up to 59.5 % and 74.9 %, respectively). In taxonomic diversity of the order *Lactobacillales*, mainly involved in ensilaging, there were genera *Lactobacillus* (up to 39.6%), *Enterococcus* (up to 36.36 %), *Lactococcus* (up to 14.4 %), *Pediococcus* (to 1.45 %) and the family *Leuconostocaceae* (to 3.52 %). Interestingly, in the silage there were the bacteria of phylum *Bacteroidetes*, families *Ruminococcaceae*, *Lachnospiraceae* and *Selenomonadales* considered the common inhabitants of the mammals' gastrointestinal tract, and also the uncultured and pathogenic microorganisms. Particularly, these were 15 genera of family *Enterobacteriaceae*, including genera *Klebsiella*, *Salmonella*, *Yersinia*, etc., among which the dangerous mammalian pathogens are frequent. On days 3 and 7, the phylum *Bacteroidetes* prevailed (59.53 and 48.91 %, respectively). On days 14 and 30, the phylum *Firmicutes* was dominant (up to 74.85 %) with the facultative aerobic bacteria of order *Lactobacillales* mostly found (up to 74.76 %). Using NGS, a total of 70 genera were attributed in the plant phyloshere, and in the silage there were 84 genera on day 3, 96 genera on day 7, 51 genera on day 14, and 69 genera on day 30. Classical microbiology methods are not enough to detect these bacteria among silage microbiota.

Keywords: silage microorganisms, epiphytic microflora, NGS-sequencing, organic acids.

The Russian Federation holds the first place in the world for the amount of silage production (30-40 million tons per year) [1]. Providing own safe voluminous forages (silage, haylage, grain haylage, hay) determines the quality of milk, which is essential for the production of baby food, functional

dairy products, and cheese.

Silage microbiocenosis is formed of epiphytic microorganisms present on forage crops. The composition of plant and silage microflora affects the fermentation processes in the silage with a direct impact on the biochemical indicators of silage quality [2-4]. Modern molecular techniques complement classical identification methods in microbiology [2-6]. These techniques do not require prior isolation of microorganisms, which makes it possible to detect the unculturable objects which appeared to make up 99 % of the microbiota [7].

To date, the reports on fodder plant and silage microbiota studies using molecular genetic methods are few and limited to the descriptions of composition and function of individual groups of microorganisms [8-11]. Moreover, the NGS (next generation sequencing) data on the diversity of epiphytic microflora and silage microbiocoenosis are not still reported.

We pioneered the use of NGS sequencing in studying phyllosphere and silage microbiom, and reported it to be rather rich in its genus diversity (which is in contrast with conventional understanding) and the presence of pathogenic and uncultured bacteria. Among others, bacteria belonging to phylum *Bacteroidetes* and families *Ruminococcaceae*, *Lachnospiraceae* and *Selenomonadales* that are considered typical inhabitants of mammal gastrointestinal tract have been found in silage.

Our purpose was to analyze the composition and structure of cocksfoot phyllosphere and silage bacterial community during maturation of silage using NGS sequencing.

Technique. Cocksfoot (*Dactylis glomerata* L.) first cut (shooting stage, humidity of 84.7 %) vegetation was used in model laboratory ensilaging with AIV 2000 Plus chemical preservative (a mixture of formic, propionic, and benzoic acids) (KEMIRA OYJ, Inc., Finland) at a dose of 4 ml/kg. Plant biomass (320 g) was stored in vacuum plastic bags in a thermostatic room at 26±1 °C.

Total DNA was isolated using Genomic DNA Purification Kit (Fermentas Inc., Lithuania) according to manufacturer's recommendations. DNA concentration in the solution was measured at a Qubit (Invitrogen Inc., USA) fluorimeter with a Quant-iT dsDNA Broad-Range Assay Kit (Invitrogen Inc., USA) according to manufacturer's instruction. PCR was performed using a Verity DNA amplificator (Life Technologies Inc., USA), and eubacterial (IDT) 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) primers flanking a 16S rRNA V1V4 gene region. A 16S rRNA gene fragment metagenomic sequencing was performed using a MiSeq system (Illumina Inc., USA; error of 5 %) with MiSeq Reagent Kit v3 (Illumina Inc., USA). The maximum length of the sequences obtained was 2×300 bp. Chimeric sequences were excluded from analysis by USEARCH 7.0 program (http://drive5.com/usearch/). Read processing by a CLC Bio GW 7.0 bioinformatics platform (Qiagen N.V., Netherlands) included overlap analysis, filtration for quality (QV > 15), and primer trimming. Estimation of microbial taxonomic affiliation up to the genus was performed using the RDP Classifier program (https://rdp.cme.msu.edu/classifier/classifier.jsp).

Mathematical and statistical processing of the results (multiple correlation and analysis of variance) was performed using Microsoft Excel 2010. The Simpson dominance index and Shannon biodiversity index were calculated for microbiological ecosystem estimation [12].

Results. Our results demonstrated that silage bacterial community differed dramatically from that in phyllosphere and varied greatly in the course of successive changes during maturation of silage.

The phyllosphere and silage microbiocenosis were found to be abundant in its genus composition, in contrast with the traditional understanding [2-6].

We identified 70 bacterial genera in the cocksfoot epiphytic microflora, and from 51 to 96 genera in silage. Maximum species diversity of silage microbiological ecosystem was observed at days 3-7, probably due to pH close to neutral or weakly acidic with a high redox potential in the early stages of silage maturation which resulted in the favorable conditions for the development of most microorganisms [2-4].

D. glomerata L. epiphytic microflora mostly included the members of phylum *Proteobacteria* (94.08 %), these are bacteria belonging to family *Entero-bacteriaceae*, genus *Pseudomonas*, and order *Burkholderiales*. Genera *Serratia* and *Pantoea* were predominant in the *Enterobacteriaceae* family. These results are somewhat contradictory to the traditional idea [13, 14] of genus *Erwinia* as the dominant family *Enterobacteriaceae* representative as a part of epiphytic microflora.

1. Bacterial community structure (taxon frequency, %) **in cocksfoot (*Dactylis glomerata* L.) phyllosphere and silage at different stages of maturation in laboratory experiments**

Taxon	Phyllosphere	Maturation of silage, days			
		3	7	14	30
Phylum *Firmicutes*	0.20	20.70	22.20	71.30	74.90
order *Lactobacillales*	0.07	0.02	7.80	71.30	74.80
uncultured *Lactobacillales*	–	–	0.06	0.11	0.07
family *Lactobacillaceae*	–	0.01	1.50	40.20	32.70
genus *Lactobacillus*	–	0.01	1.50	39.60	31.20
genus *Pediococcus*	–	–	0.07	0.55	1.50
family *Enterococcaceae*	0.04	0.01	1.90	12.40	36.40
family *Streptococcaceae*	–	–	4.00	14.40	3.90
family *Leuconostocaceae*	0.03	–	0.31	3.50	1.60
uncultured *Lactobacillaceae*	–	–	–	0.77	0.21
family *Bacillaceae*	0.12	–	0.04	–	0.08
genus *Staphylococcus*	0.02	–	–	–	–
family *Clostridiaceae*	–	0.65	0.45	–	–
family *Ruminococcaceae*	–	10.90	7.40	–	–
family *Lachnospiraceae*	–	1.30	1.00	–	–
uncultured *Clostridiales*	–	4.40	3.20	–	–
order *Selenomonadales*	–	3.40	2.30	–	0.01
genus *Erysipelothrix* sp.	–	0.04	–	–	–
Phylum *Actinobacteria*	0.06	–	–	0.01	0.14
Phylum *Proteobacteria*	94.10	0.03	14.30	26.60	20.60
family *Enterobacteriaceae*	67.60	0.01	14.30	26.50	20.20
genus *Pseudomonas*	23.40	–	–	0.01	0.06
order *Rhizobiales*	0.08	–	0.01	0.04	0.10
order *Burkholderiales*	3.00	0.02	0.01	0.02	0.24
Phylum *Bacteroidetes*	2.30	59.50	48.90	0.18	0.20
Unidentified bacteria	0.34	7.80	11.30	0.21	0.01
Other	3.00	12.00	3.30	1.70	4.20
Total genera	70	84	96	51	69

N o t e. Dashes mean that the number of microorganisms was below the limit of reliable quantification by NGS sequencing.

A sharp decline in pH, the conditions close to anaerobic, changes in the temperature and solids content, etc., resulted in radical shifts in the structure of microbiocenosis. At ensilage days 3 and 7, the members of phylum *Bacteroidetes* (59.53 and 48.91 %, respectively) were predominant in the bacterial community. An increase in the number of silage phylum *Bacteroidetes* bacteria indicates a deviation from the optimal ensilaging process, since these microorganisms have saccharolytic properties and are capable of utilizing polysaccharides, especially starch [15-17]. With proper ensilaging, epiphytic lactic acid bacteria begin to propagate actively, accumulating exponentially and occupying a dominant position at days 3 to 5 of ensilaging [2-6]. Probably, the preservative organic acid mixture inhibited the growth of epiphytic lactic acid bacteria which resulted in a natural increase in the number of undesirable microorganisms.

Classical microbiological methods do not allow identifying phylum *Bacteroidetes* bacteria in silage [2-6], while these bacteria have been traditionally considered as typical mammal GIT inhabitants [15-17]. Special attention should be paid to the substantial number of other typical mammal GIT inhabitants in silage [15-17], such as the representatives of families *Ruminococcaceae* (up to 10.88 %) and *Lachnospiraceae* (to 1.33 %), and order *Selenomonadales* (up to 3.36 %). Family *Ruminococcaceae* and *Lachnospiraceae* bacteria use some monosaccharides as the main sources of nutrients in addition to cellulose, cellobiose and xylan, competing for the substrate with lactic acid bacteria. The presence of order *Selenomonadales* bacteria is also undesirable for ensilaging as they are capable of fermenting organic acids counteracting a pH decrease [15-17]. In classical microbiology, these taxa have not been described in silage [2-6].

The members of phylum *Firmicutes* (to 74.85 %) were predominant at days 14-30, the major proportion of the latter was the facultative anaerobic lactic acid bacteria belonging to order *Lactobacillales* (to 74.76 %). Prevalence of lactic acid bacteria in the later stages of succession was due to their resistance to pH reduction to 3.0-3.5 [18], which makes them extremely competitive under the conditions of the ecosystem analyzed. These bacteria play a key role in the silage microflora formation since they produce lactic acid as the main metabolic product [19], thereby reducing pH and inhibiting the undesired microflora [2-6].

Taxonomic diversity of order *Lactobacillales* was presented by genera *Lactobacillus* (to 39.60 %), *Enterococcus* (to 36.36 %), *Lactococcus* (to 14.40 %), *Pediococcus* (to 1.45 %), family *Leuconostocaceae* (to 3.52 %), and by uncultured forms (up to 0.77 %). Our results are consistent with published data [8, 20-22]. Thus, DNA sequencing of 161 lactic acid bacteria isolates from rice silage [8] demonstrated 24 % of *Lactobacillus plantarum*, 22 % of *Lactococcus lactis*, 20 % of *Leuconostoc pseudomesenteroides*, 11 % of *Pediococcus acidilactici*, 11 % of *Lactobacillus brevis*, 7 % of *Enterococcus faecalis*, 3 % of *Weissella kimchii*, and 2 % of *Pediococcus pentosaceus*.

The number of family *Enterobacteriaceae* bacteria in the community metagenome in silage was from 0.01 to 26.49 %, while in the cocksfoot epiphytic microflora it was 67.58 %, which is significantly higher. The family *Enterobacteriaceae* bacteria are undesirable for silage as, while fermenting carbohydrates, they compete with lactic acid bacteria for nutrition sources and resist the pH reduction. A total of 15 genera of family *Enterobacteriaceae*, including genera *Klebsiella*, *Salmonella*, *Yersinia*, etc., were identified in the cocksfoot silage microbiological ecosystem, among which dangerous mammalian pathogens are frequent. The presence of family *Enterobacteriaceae* bacteria at ensilaging day 3 was insignificant (those microorganisms were probably inhibited by organic acids), but after day 7 their number increased sharply until day 14. Bacteria of the family *Enterobacteriaceae* have been described in silage long ago [5, 23, 24]. We also found uncultured microorganisms in silage. Thus, the proportion of uncultured representatives of order *Clostridiales* at ensilaging days 3 and 7 was 4.39 and 3.19 %, respectively. The presence of *Clostridiales* bacteria is undesirable in ensilaging since they are capable of cleaving organic acids and protein in addition to sugars [2-6].

The proportion of phylum *Bacteroidetes*, families *Ruminococcus* and *Lachnospiraceae*, and order *Selenomonadales* bacteria, as well as of uncultivated order *Clostridiales* representatives was the greatest at ensilaging days 3-7 and decreased dramatically from day 14 to day 30. This is probably due to the lower pH threshold of 4.5-5.0 in the most members of these taxa [15-17].

Minor (less than 1 %) silage taxa were the representatives of families *Clostridiaceae* and *Bacillaceae*, phylum *Actinobacteria*, genus *Pseudomonas*, and

orders *Rhizobiales* and *Burkholderiales*, while significant numbers of the representatives of genus *Pseudomonas* and order *Burkholderiales* (23.40 and 3.02 %, respectively) were found among the epiphytic microflora. Probably, a low redox potential and decreased pH had an inhibitory effect on those microorganisms

Furthermore, members of genus *Staphylococcus* pathogenic for mammals were found among epiphytic microflora, and genus *Erysipelothrix* bacteria, previously undetectable by classical microbiology methods, were identified in silage at day 3 [2-6].

Thus, phyllosphere and silage microbial communities' compositions were characterized by a considerable taxonomic abundance, which was confirmed by the Shannon and Simpson diversity indices (Table 2).

2. Characteristics of bacterial community diversity in cocksfoot (*Dactylis glomerata* L.) phyllosphere and silage at different stages of maturation in laboratory experiments

Index	Phyllosphere	Silage, days			
		3	7	14	30
Shannon index	1.80	2.60	2.90	2.00	2.00
Simpson index	0.76	0.86	0.90	0.80	0.79

At the days 3 and 7 of maturation, the H Shannon index was the highest (2.6 and 2.9) which indicates the greatest uncertainty and heterogeneity of the microbiocenosis composition compared to epiphytes. The greatest microorganism species diversity (84 and 96 genera, respectively) and the highest D Simpson index, characterizing dominance, also demonstrated accumulation of entropy and specific microbial community disruption in these ensilaging periods. Based on the values of environmental indices, the structure of silage biocenosis was characterized by a slightly greater homogeneity at days 14 and 30. Therefore, some stabilization of microbiocenosis took place during silage maturation.

Thus, molecular genetic methods for the analysis of cocksfoot (*Dactylis glomerata* L.) phyllosphere and silage microbiocenosis structure at different stages of maturation have allowed for the first time to explore all of the microbial diversity of this microbiosystem. According to our results, silage bacterial community structure differed dramatically from phyllosphere microorganism composition and changed significantly in succession in the cource of silage maturation. Contrary to the traditional concepts, the structure of phyllosphere and silage microbiocenosis was characterized by highly abundant taxonomic diversity. Taxa playing an important ecological role and not detected earlier by classical microbiology methods have been identified among epiphytic microflora and in silage by NSG sequencing.

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