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FERMENTATION PROCESSES IN ALFALFA HAYLAGE WITHOUT ADDITIVES AND WITH INTRODUCTION OF *Lactobacillus plantarum* STRAIN

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Abstract

The optimal pH required for the functioning of proteases in alfalfa is lower than that of meadow clover or cereal grasses, and this culture is rich in protein and pectin which is not favorable for high-quality feed production. It is recommended to accelerate the acidification of the alfalfa being hayed by adding preparations of lactic acid bacteria. In the present work, for the first time in Russia, a diversity profile of haylage microbiota during fermentation was revealed using NGS sequencing. The work aimed to study the peculiarities of alfalfa fermentation during haylage with and without using Biotrof, a lactic acid bacteria-based preparation. The experiments were performed in 2018-2019. In the first experiment, the peculiarities of biochemical and microbiological processes during alfalfa haylaging were examined. Alfalfa *Medicago sativa* L. nothosubsp. *varia* (Martyn) Arcang cv. Pastbishnaya 88 was grown (experimental field, the Williams Federal Research Center for Forage Production and Agroecology, Moscow Province), cut for hay, dried in swaths for 7 hours to a dry matter content of 43.5 % and put into 0.5 l glass vessels for haylaging. The pH dynamics, ammonia, sugar and fermentation acid levels were measured on days 0, 4, 7, 14, 28, 60, and 90 of storage. The composition of the microbial community of the plant biomass and the alfalfa haylage was analyzed in dynamics using NGS sequencing according to a modified technique. In the second series of experiments, the effect of the preparation of lactic acid bacteria Biotrof (OOO Biotrof, Russia) based on *Lactobacillus plantarum* No. 60 on storability and biochemical parameters of the haylage from alfalfa cv. Pastbishnaya 88 biomass dried to a dry matter content of 47.6 and 51.3 %, was studied. The biomass was put into 0.5 l-containers equipped with devices for measuring evolved gases for two treatments, with no additives and upon introduction of the Biotrof preparation in the recommended dose (10^5 CFU/g green mass). It was shown that a short-term wilting of alfalfa biomass to the haylage moisture resulted in 0.03-0.04 % ammonia and 0.08 % butyric acid concertation followed by an increase to 0.08-0.09 and 0.13-0.14 %, respectively, when haylaging. During wilting and early fermentation, the sugar contents in the biomass increased noticeably. In addition, the wilted alfalfa accumulates at least 3.7 % of malic acid which, like sugar, can be fermented by lactic acid bacteria. Butyric acid producers, the bacteria of the *Clostridiaceae* family, were not detected during fermentation. During haylage storage, among the bacteria of the *Clostridia* class the typical rumen microorganisms were identified of the families *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae*. We have found a relationship between an increase in the abundance of bacteria of the genus *Ruminococcus* and an increase in the amount of malic acid ($r = 0.80$, $p \le 0.05$), and also between an increase in the amount of malic acid and an increase in the number of bacteria of the phylum *Bacteroides* in the haylage ($r = 0.84$, $p \le 0.05$). The accumulation of malic acid improved the fermentability of plant biomass, causing a rapid acidification of the feed to pH 4.4-4.3 due to the introduced preparation of lactic acid bacteria Biotrof. This method improved the biochemical parameters of the feed, contributing to a decrease in the butyric acid level, however, it did not lead to a noticeable improvement in the preservation of nutrients and an increase in the energy nutritional value of the dry matter of the

obtained haylage due to the favorable fermentation process in dried alfalfa biomass. Acceleration of the acidification of the dried mass with the Biotrof preparation did not have a significant effect on the reduction of ammonia formation during fermentation. *Staphylococcus arlettae*, *Salmonella subterranea*, *Streptococcus gordonii*, and *Enterococcus cecorum* capable of causing diseases in humans and animals, survived up to 4-14 days of storage in haylage without additives. In this regard, the stored haylage, if technological disturbances occur, may contain pathogens of farm animals, therefor, antimicrobial biologicals are required for conservation. Therefore, the main effect of the Biotrof application was reduced only to an improvement in the biochemical parameters of the feed without leading to a noticeable increase in its preservation.

Keywords: alfalfa, haylage, proteolysis, microbiota, biologicals, lactobacteria, acidification, feed quality, NGS sequencing, quantitative PCR

Alfalfa is a non-silage plant because it is low in sugar and has a high buffering capacity [1]. The biological characteristics of this species have a significant influence on the result of preservation. The optimal pH required for the functioning of proteases in alfalfa is lower than in meadow clover or cereal grasses [2]. Moreover, the main proteases, under the influence of which most of the protein contained in alfalfa is hydrolyzed to non-protein nitrogen, exhibit maximum activity at pH 4.0 [3], which is due to intense proteolysis in the silage mass even in the case of rapid creation of the necessary active acidity in it [4]. The presence of a large amount of protein and pectin in alfalfa does not contribute to obtaining high-quality silage. The content of the latter in the dry matter of leaves and stems of plants reaches 10-12 and 6-9%, respectively [5]. Even in alfalfa wilted to a dry matter content of 30-35%, there is a significant amount of water weakly bound to protein and pectin, which, against the background of slight acidification of the feed, contributes to the development of bacteria in it that carry out putrefactive fermentation. For this reason, alfalfa is more often used for haylage, wilting plants to a dry matter content of 45-50% [6]. However, due to the above-mentioned reasons, a certain amount of butyric acid can still accumulate in the feed [7]. To prevent this, it is recommended to accelerate the acidification of the alfalfa being hayed by adding preparations of lactic acid bacteria [8].

Since during the haylage making of alfalfa, along with the necessary wilting of plants, the degree of acidification plays a role, the methods aimed at improving the fermentability of the dried mass are important. According to the available data, it is possible to increase the sugar content in the dry matter of alfalfa and, therefore, to improve its fermentability due to short-term (4-8 h) wilting in swaths to the haylage moisture content [9]. However, the mechanism of this process has not yet been fully understood. The reasons for the increase in sugar content in alfalfa haylage making are not fully understood, although this phenomenon has been known for a long time. There is no ultimate clarity regarding the formation of butyric acid in alfalfa haylage making. Some authors argue that in this case, butyric acid does not accumulate at all, despite the presence of clostridium spores in the dried mass [10].

Some researchers believe that the safety of haylage is associated exclusively with the phenomenon of physiological dryness, which inhibits the development of putrefactive microflora [11, 12]. At present, in Russia, the studies of the microflora of preserved feed using molecular methods are carried out only in the laboratory of OOO BIOTROF (St. Petersburg). Abroad, the research works are devoted to the analysis of the microbiocenosis of silage [13, 14]. This may be due to the problem of obtaining pure DNA material of haylage microflora because of to the presence in the biomass of a large number of organic impurities (polysaccharides, various organic acids, degradation products of protein and fats, nucleases, etc.) [15], which can reduce the quality of DNA purification [16].

Earlier, for the first time in Russia, we optimized the procedure of DNA extraction for microbial community of haylage [17]. Using terminal restriction fragment length polymorphism and quantitative polymerase chain reaction (qPCR) analysis, we studied the microflora composition of alfalfa haylage, dried to a dry matter content of 55±1.9%, on day 30 of storage. The total number of bacteria in the haylage microflora was $1.1\times10^8\pm3.4\times10^6$ genomes/g. The number of phylotypes of microorganisms represented in the haylage was 58±3.9, the average Shannon index was 3.3±0.22, and Simpson's index was 0.96±0.05. These results demonstrated the complexity of organizing microbial communities in haylage.

In our opinion, the further study of the microbial ecosystem of haylage in dynamics is extremely interesting. Such an ecosystem is constantly changing and subject to anthropogenic interference, which makes it a unique and complex ecological niche.

In this work, for the first time in Russia, the diversity of the composition of microorganisms during the fermentation of haylage was revealed using next-generation sequencing (NGS). During the fermentation, no typical silage microorganisms, producers of butyric acid of the *Clostridiaceae* family, were found. Among the bacteria of the *Clostridia* class in the haylage during storage, typical rumen dwellers were identified, i.e., bacteria of the families *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae*. For the first time, a relationship was revealed between an increase in the abundance of bacteria of the genus *Ruminococcus* and an increase in the amount of malic acid, as well as between an increase in the level of malic acid and an increase in the number of bacteria of the phylum *Bacteroides*.

This work aimed to investigate the peculiarities of fermentation and the structure of the microbiome for convenient alfalfa haylaging and using the preparation Biotrof based on lactic acid bacteria.

Methods. The experiments were performed in 2018-2019. In the first experiment, the features of biochemical and microbiological processes during alfalfa haylaging were studied. The raw material used was variegated alfalfa *Medicago sativa* L. nothosubsp. *varia* (Martyn) Arcang of the cultivar Pastbishchnaya 88 (experimental field of the Federal Williams Research Center of Forage Production and Agroecology, Moscow Province). The crop was harvested in the budding phase. Before filling in 0.5 l glass bottles, alfalfa biomass was dried in swaths for 7 hours to a dry matter content of 43.5%. The dynamics of pH, the content of ammonia, sugar, and fermentation acids were assessed during the alfalfa haylage making in laboratory vessels in the usual way. The haylage was analyzed after 0, 4, 7, 14, 28, 60, and 90 days of storage. Simultaneously, the obtained feed samples were frozen at –25 °C for molecular studies.

The composition of the microbial community of the original alfalfa plant mass and haylage was analyzed using NGS sequencing with modifications [17]. Total DNA was extracted using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. When obtaining products for subsequent NGS sequencing, PCR was performed on a Veriti Thermal Cycler DNA amplifier (Life Technologies, Inc., USA) with eubacterial primers (IDT) 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTANVGGGTWTCTAAT-3′), flanking the V1V3 region of the 16S rRNA gene. Metagenomic sequencing (MiSeq system, Illumina, Inc., USA) was performed using the MiSeq Reagent Kit v3 (Illumina, Inc., USA). The maximum length of the obtained sequences was 2×300 bp. Chimeric sequences were excluded from analysis using the

USEARCH 7.0 program (http://drive5.com/usearch/). Processing of the obtained 2×300 bp reads was performed using the CLC Bio GW 7.0 bioinformatics platform (Qiagen N.V., the Netherlands) and included detection of overlapping sequences from forward and reverse primers for unambiguous sequence reading, quality filtering (QV > 15), and primer trimming. The taxonomic affiliation of microorganisms to genera was determined using the RDP Classifier program (https://sourceforge.net/projects/rdp-classifier/).

qPCR (real time PCR — RT-PCR) was performed using a DTlite-4 detecting amplifier (OOO NPO DNA-Tekhnologiya, Russia) with a set of reagents for RT-PCR and primers HDA1 (5′-ACTCCTACGGGAGGCAGCAG-3′) and HDA2 (5′-GTA-TTACCGCGGCTGCTGGCA-3′) in the presence of an intercalating dye EVA Green (ZAO Syntol, Russia). The amplification protocol was as follows: 3 min at 95 °C (1 cycle); 1 min at 95 °C, 1 min at 57.6 °C, 1 min at 72 °C (40 cycles); 5 min at 72 °C (1 cycle).

The diversity of the bacterial community was assessed graphically as a heatmap constructed using the "pheatmap" package Version: 1.0.12 for R (https://cran.r-project.org/web/packages/pheatmap/pheatmap.pdf). Hierarchical clustering by samples was performed according to Ward's method on a matrix built according to the Euclidean distances.

In the second series of experiments, we studied the effect of the Biotrof preparation (OOO Biotrof, Russia) based on *Lactobacillus plantarum* No. 60 on the safety and biochemical parameters of haylage from alfalfa cultivar Pastbishnaya 88 when wilted to a dry matter content of 47.6 and 51.3%. The haylage was prepared in laboratory 0.5 l containers equipped with devices to measure volumes of the emitted gases when haylaging without additives and with the introduction of the Biotrof preparation in the dose recommended by the manufacturer (105 CFU/g of green mass).

The dry matter content in the green biomass and the resulting feed was determined by drying the weighed portions at 105 °C to constant weight, the sugar concentrations were measured by the Bertrand method, ammonia according to Longi, pH with an I-500 potentiometer (Russia), organic acids (lactic, acetic, butyric, formic, propionic, succinic, malic, citric, tartaric, and oxalic) by capillary electrophoresis (KAPEL-105M, Lumex, Russia).

The mathematical and statistical processing of the results was performed by standard methods of analysis of variance in Microsoft Excel XP/2003, PAST (http://priede.bf.lu.lv/ftp/pub/TIS/datu_anali-ize/PAST/2.17c/download.html), and R-Studio (https://rstudio.com). The results are presented as the mean (M) and standard errors of the mean (±SEM). The differences were assessed using Student's t-test. The results were considered statistically significant at $p \leq 0.05$.

Results. It was found that even during the 7-hour wilting of alfalfa in swaths, up to 0.03% of ammonia and 0.08% of butyric acid accumulated in its dry matter (Table 1). The main reason for the accumulation of ammonia was protein hydrolysis under the influence of plant enzymes, followed by deamination of the formed amino acids.

It is important, however, to note that with brief wilting of alfalfa to haylage moisture, the accumulation of ammonia in its dry matter did not increase in comparison with its content in the dry matter of freshly cut alfalfa. This indicates that, along with the breakdown of protein during wilting of alfalfa, synthetic processes take place, in which ammonia is consumed for forming amides. The latter, as it is known, occurs under the condition that high intensity of respiration remains in the dried mass.

1. Biochemical parameters of haylage from alfalfa *Medicago sativa* L. cv. Pastbishchnaya 88 (wilted to 43.5 % dry matter) during storage (lab test)

Parameter	Storage						
	0 day	4 days	7 days	14 days	28 days	60 days	90 days
Content in dry matter, %:							
ammonia	0.03	0.05*	0.05	0.09*	0.08	0.09	0.09
sugar	4.52	5.24*	4.01*	5.55*	4.45*	2.28*	1.25*
organic acids							
lactic	0.09	0.76*	1.00	0.21*	0.61*	4.17*	6.09*
acetic	0.05	0.35*	0.13*	0.21*	0.19	0.34*	0.45*
butyric	0.08	0.13*	0.13	0.14	0.11	0.11	0.10
succinic	0.09	0.15*	0.15	0.20	0.19	0.24	0.34*
malic	3.72	3.90	3.19*	3.89*	3.12*	2.05*	2.03
citric	0.48	0.48	0.50	0.48	0.45	0.34	0.19*
pH	6.18	5.92*	5.93	5.95	5.87*	5.32*	4.93*

* Differences with the indicator in the previous time period is statistically significant at $p \leq 0.05$.

There are grounds to believe that a rather significant increase in the sugar content in the dry matter of the dried alfalfa biomass is associated with synthetic processes. This phenomenon is still explained by the hydrolysis of starch contained in plants under the influence of enzymes. However, this statement is not supported by the available experimental data. In particular, it was found that, during wilting, plants accumulated not maltose, as would be expected during starch hydrolysis, but sucrose, which is the main product of photosynthesis [18]. Based on the generally accepted point of view, the authors explain this by the fact that when starch decomposes, sucrose is formed not primarily, but by a secondary path, that is, as a result of its subsequent synthesis from glucose and fructose. Also, the available data show that such a process occurs only when alfalfa is dehydrated in the sun in swaths and is not initiated when plants wilt in the dark [9]. This is evidence that the sugar content during short-term wilting of alfalfa increases as a result of photosynthesis, which also takes place in the mowed mass for some time.

The initial and not entirely correct interpretation of the discussed issue, obviously, was formed on the basis of the obtained data. In particular, it was found that photosynthesis slowed down when plants lost more than 15-20% of moisture, while respiration proceeded intensively even with a higher degree of wilting of plants [19]. From this, it was quite possible to conclude that the slowed-down photosynthesis combined with intensive respiration cannot provide a noticeable increase in the sugar content in the dry matter of the wilted biomass.

However, it became known that with intense dehydration, plant growth first stops, then photosynthesis is inhibited, and only then the respiration of plant cells is suppressed [20]. It is the cessation of plant growth, which causes a delay in the outflow of the sucrose formed during photosynthesis to other organs, including the root system already torn away from them, that contributes to a noticeable increase in the sugar content in the vegetative mass. This phenomenon is of a protective nature and is of fundamental importance when growing plants in drought conditions. Despite the cessation of the growth of the vegetative mass, the root system (and above all its growth zones) is still in rather favorable conditions and uses the excess sugar formed in the leaves for its own enhanced growth, leading to the reclamation of deeper and, therefore, more waterlogged layers of soil.

As it was noted above, with intensive wilting on haylage, alfalfa still retains a high respiration rate due to the adaptive restructuring of the respiratory apparatus to work under conditions of dehydration. This, in particular, is indicated by the high accumulation of dried mass of malic acid in the dry matter, which allows plants to synthesize citric acid directly from malic acid [21]. The described mechanism makes it possible to reduce the dependence of plants on such a costly process as glycolysis, while simultaneously ensuring the work of the Krebs cycle in a shortened type. Meanwhile, alfalfa, wilted to the specified dry

matter content, already suffers from a lack of oxygen. This, in particular, is indicated by the accumulation of a certain amount of succinic acid in it. The appearance of the latter indicates its release from mitochondria and serves as an indicator of the development of progressive hypoxia in the mass [22]. During normal plant respiration, succinic acid is either not detected or is determined in trace amounts, since it is formed only in mitochondria, where it is instantly utilized. Obviously, the accumulation of a certain amount of butyric acid in the dried mass of alfalfa is also associated with the onset of hypoxia. There are reports in the literature [18] indicating the possibility of butyric acid synthesis in plants with a lack of oxygen due to a violation of fat metabolism. Malic acid, or malate, is formed as a result of the glycolytic decomposition of starch, which is very quickly synthesized in the light from sucrose formed during photosynthesis and is deposited in chloroplasts [23]. This explains the fact that when alfalfa is wilted in the dark, that is, in the absence of photosynthesis, neither sugar nor malic acid is formed in it [9].

An increase in the content of sugars, and, consequently, an improvement in the fermentability of alfalfa was observed in the first 4 days of its haylage making. As it is while wilting, this phenomenon was aimed at preserving the vital activity of plants in the conditions of the onset of anaerobiosis, but was conducted due to the use of their own reserve nutrients. According to the available data [24], the main source of sugar in this case is the hydrolysis of hemicelluloses, which occurs under the influence of plant enzymes.

From the obtained results, it follows that this process proceeded most vividly at the very beginning of alfalfa haylage making, when there was still no noticeable fermentation in it associated with the accumulation of organic acids. As the intensity of lactic acid fermentation increased at the final stages of haylage making, the sugar content decreased markedly.

Along with an increase in the content of monosaccharides, an important condition for improving the fermentability of alfalfa is the possibility of using malic acid by lactic acid bacteria, the accumulation of which in the dry matter of the wilted mass was only slightly inferior to the sugar content. Our studies have shown that, as in the case of monosaccharides, malic acid fermentation took place only at the later stages of haylage making, when the content of lactic acid in the feed increased markedly. From this, it can be concluded that the main condition for the fermentation of both malic acid and monosaccharides was the provision of developing intensive lactic acid fermentation in the feed. The same can be stated about citric acid found in plants.

According to the data we obtained, a significant ($p \leq 0.05$) increase in the ammonia content in dry matter of the haylage mass was noted only in the first 2 weeks. Then its amount stabilized and did not change during the entire subsequent storage period of the feed. In protein degradation during haylage and silage making form dried alfalfa mass, researchers usually distinguish two main stages [25]. At the first stage of fermentation of the biomass, plant enzymes are mainly active, causing the breakdown of protein to free amino acids. The main role in the deamination of amino acids, that is, in the accumulation of ammonia as such, is assigned to microbial enzymes.

Some authors note that a certain amount of ammonia can be formed as a result of the influence of plant enzymes on the protein [18]. They came to this conclusion on the basis of the results of experiments on ensiling meadow clover with toluene. The latter is known to inhibit the development of bacteria without having a noticeable negative effect on the activity of enzymes. It was found that in the absence of bacterial development in the feed, protein decomposition was accompanied by the accumulation of a certain amount of ammonia in the mass,

the nitrogen of which was up to 5.0% of the total feed nitrogen. It is not excluded, however, that in this case the addition of toluene, causing the death and lysis of bacterial cells, promoted the release of the enzymes contained in them into the external environment. In other words, both plant and microbial enzymes were involved in proteolysis.

In the first 4 days of haylage making, a significant ($p \leq 0.05$) increase was noted in the content of butyric acid in the dry matter of the feed, which increased 1.6 times compared to the accumulation of the original dried alfalfa in the dry matter. The fact that butyric acid was formed at the very beginning of the haylage making of alfalfa, when no noticeable fermentation was yet observed in it, indicates that, as while wilting, some amount of butyric acid can be formed in a purely biochemical way, i.e., without the participation of microbes.

Thus, Maevskii et al. [26], relying on the results obtained in the experiments on animals, note that under conditions of hypoxia, the amount of reduced nicotinamide adenine dinucleotide (NADH) in mitochondria noticeably increases, which leads to a decrease in the oxidation of NAD-dependent substrates. As a result, there is an excessive accumulation of acetyl-CoA, a product of β-oxidation of fats, the further oxidation of which is inhibited due to the fact that an increase in NADH causes a rapid restoration of oxaloacetate to malate. As a result, acetyl-CoA remains without a partner, which is necessary to enter the Krebs cycle, and instead of complete oxidation, it becomes a source of ketone bodies, fatty acids, and cholesterol formation. There are researchers who believe that, when making silage from dried alfalfa, along with proteolysis, the lipolysis process actively proceeds in it, the main cause of which is plant, rather than microbial, lipase [27].

For a more detailed study of ammonia and butyric acid formation during alfalfa haylage making, we determined the dynamics of the total number and species composition of microorganisms by the storage period of the feed.

qPCR analysis showed that the total number of bacteria in the haylage at all periods ranged from $1.5\times10^6\pm9.3\times10^5$ to $2.0\times10^7\pm1.1\times10^6$ cells/g (Table 2). That is, the alfalfa haylage at all stages of storage was quite significantly contaminated with microorganisms, which indicates a fairly active microbiological fermentation and contradicts the data on the absence of microbial fermentation processes in the haylage [11, 12]. Nevertheless, comparing the obtained data with our previous results on silage [17], it should be noted that the fermentation processes in haylage are less active. The authors have shown that the total content of bacteria in the silage ecosystem during the conservation of perennial grasses ranged from 5.2×10^7 to 99.4×10^9 genomes/g.

However, the composition of the haylage microflora sharply differed from the composition of epiphytic microorganisms of the original plant mass of alfalfa and changed in the course of successional changes occurring during storage (see Table 2). Probably, these changes occurred as a result of creating anaerobic conditions and changes in the biochemical composition of the feed. Thus, the composition of the haylage microflora was represented by 8 phyla of microorganisms (Fig.), while the structure of the epiphytic microflora included 18 phyla of bacteria.

Typical [28] microorganisms of the phylum *Acidobacteria* significantly dominated on the mowed alfalfa plant mass (62.6 ± 3.4 % at $p \leq 0.05$) (see Fig.). Despite their widespread prevalence in the environment, knowledge of the metabolism of these bacteria is in its infancy due to the almost complete impossibility of cultivation on nutrient media. The information on the presence of these bacteria in the epiphytic microflora of plants was obtained only after the appearance of methods for analyzing the sequences of 16S rRNA genes [29].

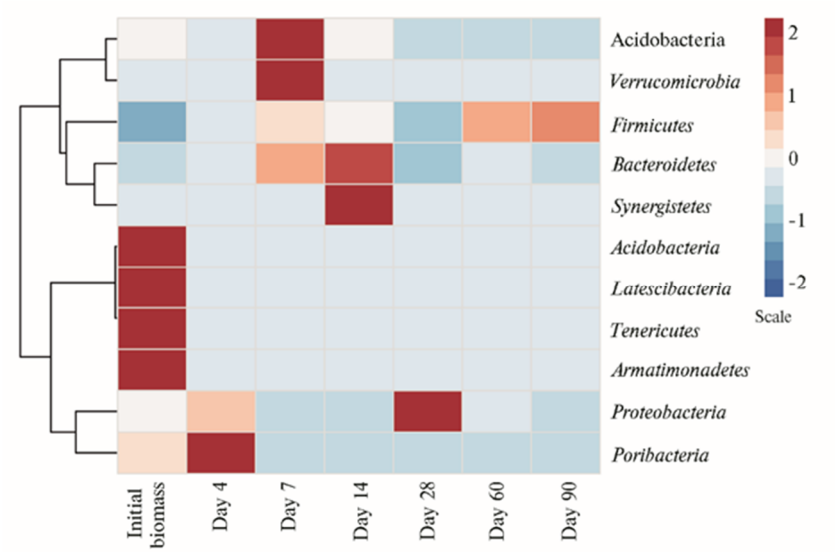
2. Microbial community composition in haylage from alfalfa *Medicago sativa* L. cv. Pastbishchnaya 88 (wilted to 43.5% dry matter) during storage (M±SEM, n = 3, lab test)

Taxon	Original biomass	Storage					
		4 days	7 days	14 days	28 days	60 days	90 days
		dPCR analysis, cells/g					
Total counts	$3.3\times10^8\pm1.4\times10^7$	$2.0\times10^7\pm1.1\times10^6$*	$9.0\times10^6\pm2.1\times10^5$*	$4.2\times10^6\pm5.6\times10^5$	$1.15\times10^7\pm9.9\times10^5$*	$1.5\times10^6\pm9.3\times10^5$*	$1.15\times10^7\pm8.9\times10^5$*
		NGS sequencing, %					
Unclassified bacteria	14.80±0.790	47.08±2.600*	50.00±3.100	53.85±3.500	35.96±1.900*	45.45±2.300*	50.91±3.900
Class *Acidobacteria*	62.56±3.400	3.64±0.210*	1.43±0.073*	0	1.12±0.059*	0	0
Class *Actinobacteria*	1.29±0.070	0.73±0.050*	4.29±0.340*	1.10±0.054*	0	0	0
Class *Aiphaproteobacteria*	2.76±0.150	0.73±0.048*	1.43±0.062	0	23.60±1.700*	0	0
Class *Anaerolineae*	0.02±0.001	0	0	0	0	0	0
Class *Aquificae*	0.02±0.002	0	0	0	0	0	0
Class *Armatimonadia*	0.02±0.002	0	0	0	0	0	0
Class *Bacilli*:	1.76±0.080	14.05±0.720*	7.14±0.360*	1.10±0.052*	7.87±0.420*	31.82±1.750*	45.45±2.600*
family *Bacillaceae*	0.37±0.020	2.74±0.150*	4.29±0.29*	1.10±0.049*	1.12±0.059	4.55±0.310*	0
family *Staphylococcaceae*	0	0	1.43±0.081*	0	0	0	0
family *Lactobacillaceae*	1.39±0.078	11.31±0.450*	4.29±0.330*	0	6.74±0.450*	27.27±1.600*	45.45±2.400*
Class *Bacteroidia*	1.16±0.063	3.47±0.190*	12.86±0.520	17.58±0.92*	0	4.55±0.380*	1.82±0.160*
Class *Betaproteodacteria*	1.45±0.081	0.18±0.009*	0	0	0	0	0
Class *Caldilineae*	0.02±0.002	0	0	0	0	0	0
Class *Caldisericia*	0.06±0.003	0	0	0	0	0	0
Class *Chthonomonadetes*	0.35±0.020	0	0	0	0	0	0
Class *Clostridia*	1.23±0.073	3.10±0.220*	15.71±0.800*	19.78±0.99*	0	9.09±0.560*	0
Class *Cytophagia*	0.31±0.030	0.18±0.007*	0	0	0	0	0
Class *Deinococci*	0.02±0.004	0	0	0	0	0	0
Class *Deltaproteobacteria*	0.06±0.004	0	0	0	0	4.55±0.290*	0
Class *Erysipelotrichia*	0.02±0.002	0	0	0	0	0	0
Class *Flavobacteria*	0.15±0.008	0.18±0.008	0	0	0	0	0

Continued Table 2

Class *Gammaproteobacteria*:	11.03±0.750	26,09±1,500*	0	2,20±0,190*	31,46±1,460*	4,55±0,300*	1,82±0,170*
order *Enterobacteriales*:	5.61±0.290	23,54±1,200*	0	1,10±0,150*	30,34±1,110*	0	0
Salmonella subterranea	0	0,36±0,019*	0	0	0	0	0
Class *Mollicutes*	0.04±0.005	0	0	0	0	0	0
Order *Selenomonadales* (class *Negativicutes*)	0.10±0.005	0,55±0,030*	4,29±0,360*	3,30±0,220*	0	0	0
Class *Opitutae*	0.02±0.003	0	0	0	0	0	0
Class *Phycisphaerae*	0.02±0.001	0	0	0	0	0	0
Class *Planctomycetia*	0.04±0.004	0	0	0	0	0	0
Class *Sphingobacteriia*	0.50±0.150	0	0	0	0	0	0
Class *Spirochaetia*	0.04±0.006	0	0	0	0	0	0
Class *Synergistia*	0.020±0.0021	0	0	1,10±0,130*	0	0	0
Class *Thermodesulfobacteria*	0.020±0.0026	0	0	0	0	0	0
Class *Thermomicrobia*	0.020±0.0015	0	0	0	0	0	0
Class *Verrucomicrobiae*	0.020±0.0023	0	1,43±0,160*	0	0	0	0

* Differences with the indicator in the previous time period is statistically significant at $p \leq 0.05$.



Heatmap analysis of bacterial community in the initial plant biomass and haylage of alfalfa *Medicago sativa* L. cv. Pastbishchnaya 88 (wilted to 43.5 % dry matter) during storage (lab test). Minor (< 0.06%) phyla *Fusobacteria*, *Spirochaetes*, *Chloroflexi*, *Aquificae*, *Planctomycetes*, *Caldiserica*, and *Thermodesulfobacteria* are not shown.

The abundance of the phylum *Acidobacteria* sharply decreased (to 3.64±0.21 at $p \leq 0.05$) already on day 4 of haylage storage, and after 28 days, these bacteria were almost completely eliminated. The obtained data are logical, since bacteria of the phylum *Acidobacteria* are oligotrophic [30], and, probably, the ecosystem of stored feed, rich in nutrients, is unfavorable for them. At the same time, representatives of the phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the dominant bacteria during the storage of haylage, which repeats the trends observed during the development of silage succession [13, 31].

Nevertheless, even after 3 months of storage, only about half of the microorganisms contained in the feed were represented by lactic acid bacteria of the *Lactobacillaceae* family (see Table 2) of the phylum *Firmicutes*, the traditional inhabitants of the silage microflora [31]. Probably, the environmental conditions typical for haylage (pH, dry matter content, etc.) were not so favorable for the vital activity of these microorganisms. It is known that lactobacteria play a decisive role in the processes of microbial fermentation of preserved feeds. They transform the mono- and disaccharides of feed with the formation of lactate, which leads to acidification of the feed and the displacement of microflora, conducting fermentation processes undesirable for haylage [32].

As a feature of fermentation of alfalfa haylage, it should be noted that there is no relationship between the accumulation of butyric acid and the number of Clostridia (class *Clostridia*) in the dried mass ($p \leq 0.05$). Thus, if the maximum amount of butyric acid in the dry matter of the feed was formed already after 4 days of fermentation, then the greatest number of Clostridia was noted only after 14 days of storage. This indicates that a significant part of the butyric acid that appears at the very beginning of alfalfa haylage has a bio-chemical origin.

Filatov et al. [33] also did not find a relationship between the accumulation of butyric acid and the number of Clostridia. The authors noted a high number of butyric acid bacteria (about 2.0×10^5 CFU) in 1 g of haylage from dried alfalfa (47.5% dry matter) during the entire 90-day storage period of the feed. However, the resulting feed did not accumulate butyric acid. This indicates that

in alfalfa haylage, clostridial spores in most cases remain inactive during the entire storage period of the feed. At present, foreign researchers also share this opinion [10]. However, this is not always the case. The authors associate the differences in the formation of butyric acid with the varietal characteristics of alfalfa, the degree of wilting of plants, on which, in their opinion, the clostridial community largely depends, and, consequently, the time of occurrence and intensity of butyric acid fermentation, as well as with other factors.

Indeed, some members of the *Clostridia* class, belonging to the *Clostridiaceae* family, produce butyric acid during fermentation, including in preserved feeds. Some of them (*C. sporogenes*, *C. bifermentans*, *C. sphenoides*) have proteolytic properties, and therefore their presence in preserved feed is undesirable [32]. However, in the authors' experiment, representatives of the *Clostridiaceae* family were detected only in the initial plant raw material of alfalfa; during fermentation, they were completely replaced by representatives of another microflora. Interestingly, among the bacteria of the *Clostridia* class, such families as *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* were found in haylage during storage. The main products of their metabolism are acetic, valeric, malic, and propionic acids, while butyric acid is formed in minor amounts, and some types of bacteria do not form it at all [34]. Interestingly, in the authors' experiment, when calculating Pearson's correlations, a significant relationship was found between an increase in the content of bacteria of the genus *Ruminococcus* and an increase in the amount of malic acid ($r = 0.8$ at $p \leq 0.05$). Bacteria of the families *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae* and *Ruminococcaceae* are representatives of the normal flora of the intestinal and cicatricial microbiocenosis [35].

There is a quite interesting fact, which is worthy of discussion, that a significant relationship was also found between an increase in the number of bacteria in the phylum *Bacteroides* and an increase in the content of monosaccharides ($r = 0.76$ at $p \leq 0.05$), butyric ($r = 0.95$ at $p \leq 0.05$) and malic ($r = 0.4$ at $p \leq 0.05$) acids. This is quite expected, since it is known that the metabolism of starch with the release of a glucose molecule [36] is the main pathway of metabolism in these microorganisms. As a result, the bioavailability of glucose can be increased for microorganisms that produce butyric and malic acids.

Consequently, a feature of alfalfa haylage is that proteolytic forms of clostridia do not receive significant development in it, that is, the feed is characterized by a favorable direction of the fermentation process.

Nevertheless, on days 4 and 28 of storage in haylage, the number of bacteria of the *Enterobacteriaceae* family increased sharply, up to 23.5±1.2 and 30.3±1.11%, respectively ($p \leq 0.05$). They are undesirable for the fermentation process of feeds, because monosaccharides become the source of their vital activity, which makes them direct competitors of lactobacteria [32]. Among the genera of enterobacteria, during storage of haylage, both typical epiphytic bacteria (*Erwinia*, *Serratia*, *Pantoea*) and pathogenic forms (*Enterobacter*, *Escherichia*, *Shigella*, *Klebsiella*, *Salmonella*) were identified.

Interestingly, microorganisms *Staphylococcus arlettae*, *Salmonella subterranea*, *Streptococcus gordonii*, *Enterococcus cecorum*, capable of causing diseases in humans and animals, survived up to 4-14 days of storage in haylage. Thus, coagulase-negative staphylococci are becoming a common cause of bacteremia [37]. They are detected in the blood of people with cardiovascular diseases. Earlier, *Staphylococcus arlettae* was isolated from the skin and manure of poultry and goats [37]. *Salmonella subterranea* is a relatively new species found in 2004 in natural sources [38]. The survival of *Salmonella subterranea* in haylage at the initial stages of fermentation is understandable [38]. *Streptococcus gordonii* is a bacterium that is recognized as the

causative agent of bacterial endocarditis and human empyema [39]. In bovine cattle, bacteria from the genus *Streptococcus* are considered pathogens associated with subclinical mastitis, many diseases of the reproductive system (abortion, stillbirth, vulvitis, vaginitis, and metritis), valvular endocarditis, and septicemia [40]. *Enterococcus cecorum* is a causative agent of joint diseases; however, to date, its pathogenicity has been proven only for poultry [41]. Consequently, stored haylage, the making of which took place in violation of technological methods, may contain pathogens of agricultural animals, which determines the need for the use of biological products with antimicrobial activity for its preservation.

The number of unidentified bacteria in the experiments ranged from 35.9±1.9 to 50.9±3.9%. These bacteria belonged to the rank of objects for the cultivation of which there are currently no nutrient media; therefore, they became known only with the development of molecular-biological methods [42]. The high proportion of unclassified microorganisms, noted in the haylage during the entire storage period, also did not lead to any significant increase in nutrient losses and deterioration of the biochemical parameters of the obtained feed. Despite the presence of these microorganisms, the authors did not observe an increase in the accumulation of ammonia and butyric acid, as well as a decrease in sugar content, a decrease in the amount of which began only after 2 months of storage of haylage, that is, after activation of lactic acid fermentation in it. It can be concluded that the introduction of lactic acid bacteria during alfalfa haylage should not be accompanied by any significant effect.

To be convinced of this, we prepared haylage from dried alfalfa without preservatives and with the introduction of lactic acid bacteria in the form of the Biotrof starter culture. The introduction of Biotrof preparation, stimulating lactic acid fermentation, caused an increase in the breakdown of nutrients to gaseous products, as a result of which the volume of gases released during haylage making increased by 1.5-2.4 times ($p \leq 0.05$) (Table 3). This was due to an increase in the fermentation of mono-sugars, the content of which in the dry matter of the feed decreased by 3.1-3.7 times ($p \leq 0.05$). Stimulation of lactic acid fermentation led to rapid acidification of the feed to the limit excluding the development of butyric acid bacteria. As a result, the accumulation of butyric acid decreased by 1.3-2.8 times in comparison with its content in ordinary haylage. However, in this case, the accumulation of butyric acid in the dry matter of the feed was 0.11-0.15%, that is, it was as much as can be formed as a result of biochemical processes.

3. Gas emission and biochemical parameters of conventional haylage of alfalfa *Medicago sativa* L. cv. Pastbishchnaya 88 and haylage added with Biotrof preparation (M±SEM, n = 3, lab test)

Biotrof preparation	Emission, l/kg dry matter of green mass	pH	Percent per feed dry matter			
			ammonia	organic acids		monosugars
				lactic	butyric	
Wilted to 47.6% dry matter						
Not added	2.39±0.430	5.59±0.010	0.23±0.010	0.30±0.040	0.20±0.010	2.26±0.090
Added	5.73±0.120*	4.31±0.010*	0.27±0.010	15.48±0.320*	0.15±0.010*	0.73±0.040*
Wilted to 51.3 % dry matter						
Not added	3.04±0.220	5.45±0.090	0.17±0.010	2.86±0.100	0.31±0.040	4.63±0.260
Added	4.62±0.120*	4.25±0.010*	0.14±0.020	14.06±0.200*	0.11±0.030*	1.25±0.040*

* Differences with the indicator for haylage without additives is statistically significant at $p \leq 0.05$.

The acceleration of the acidification of the alfalfa haylage mass under the influence of the Biotrof preparation did not lead to a significant reduction in the accumulation of ammonia in the feed. That is, the main parameter that determines the degree of protein breakdown to ammonia during silage and haylage making of

alfalfa is the degree of wilting of plants. According to the available data [33], with an increase in the dry matter content in the silage mass of alfalfa from 21.1 to 31.5; 41.5 and 52.0%, the amount of ammonia nitrogen in relation to the total nitrogen of the feed is reduced from 18.6 to 8.8; 4.5 and 4.9%. Since the use of lactic acid bacteria preparations does not lead to a noticeable improvement in the preservation of nutrients in alfalfa haylage, improving only its biochemical parameters, it does not lead to a noticeable increase in the energy nutritional value of the dry matter of the obtained feed [43]. The haylage from alfalfa treated with preparations of lactic acid bacteria has a higher productive effect [44]. The authors explain this phenomenon by an increase in the total mass of the microflora of the rumen chyme, which can be a source of protein for animals.

Thus, with short-term wilting of alfalfa to haylage moisture and at the very beginning of its haylage making, there is a noticeable increase in the sugar content in the dry matter of the green mass. In addition, malic acid accumulates in large quantities in wilted plants, which, like mono-sugars, is fermented by lactic acid bacteria. High-throughput sequencing identified typical rumen inhabitants of the families *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* among bacteria of the *Clostridia* class in haylage during storage. Interestingly, butyric acid producers, bacteria of the *Clostridiaceae* family, were not detected during fermentation. A significant relationship was shown between an increase in the content of bacteria of the genus *Ruminococcus* and an increase in the amount of malic acid, as well as between an increase in the content of malic acid and an increase in the number of bacteria of the phylum *Bacteroides*. The accumulation of malic acid led to an improvement in the fermentability of alfalfa being hayed, as a result of which, under the influence of the preparation of lactic acid bacteria Biotrof, it was quickly acidified to pH 4.4-4.3, which ensures the stability of the feed during storage. The expediency of this technique is due to the fact that in the haylage mass in large quantities (up to half of the total number of microorganisms) there are unclassified bacteria that do not grow on conventional nutrient media and have not been sufficiently studied. These bacteria do not lead to significant losses of nutrients during haylage making, however, they worsen the biochemical parameters of the feed, contributing to an increase in the accumulation of butyric acid in it. Therefore, the main effect of the use of lactic acid bacteria preparations in alfalfa haylage is reduced only to an improvement in the biochemical parameters of the feed, without leading to a noticeable improvement in its preservation. Also, *Staphylococcus arlettae*, *Salmonella subterranea*, *Streptococcus gordonii*, and *Enterococcus cecorum* were found in the haylage, which can cause diseases in humans and animals.

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