Aerobic stability of alfalfa silage and methods of its improvement

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Abstract. Alfalfa is a non-silage crop, the silage of which became possible after the effect of drying was detected. A special feature of alfalfa is the intensive proteolysis that occurs during silage, and the prolonged development of undesirable microflora due to the slow acidification of feed. The objective of the research was to determine the effectiveness of the use of Biotrof, Biotrof 111, Biotrof 2+ and Biotal Axfast NS Gold in the silage of dried (37.07% SV) alfalfa mass. It was found that the use of these drugs contributed to the rapid acidification of feed to a pH of 3.97-4.08, against a pH of 4.96 in silage without additives, which led to the suppression of the vital activity of undesirable bacteria, a decrease in the breakdown of nutrients to gaseous products by 1.7-2.3 times and the accumulation of ammonia by 1.5-4.0 times. Due to the high resistance of alfalfa silage to aerobic spoilage, the use of bacterial preparations did not lead to an improvement in the aerobic stability of the silage, but by restraining the development of some yeasts, including pathogenic ones, and fungi of the Aspergillus sp. species, it helped to improve the sanitary status of the feed. Yeasts of the genera C. gattii and D. hansenii serve as marker organisms, an increase in the number of which indicates the occurrence of aerobic spoilage in the feed.

Keywords: alfalfa, silage, dry matter content, bacterial preparations, aerobic spoilage, yeast, mold fungi, ammonia, organic acids.

1. Introduction
Alfalfa is a non-silage culture [1], which was initially used as a protein component when harvesting combined silage [2]. The improvement of the fermentability of alfalfa due to drying has opened the possibility of its silage in its pure form, but under a number of conditions. First of all, it is necessary to ensure rapid (4-8 hours) drying of alfalfa in the sun in the patches to a dry matter content of ≥ 40.0% [3], as a result of which the sugar content in its dry matter increases by 30.0%. At the same time, the content of malic and citric acids increases in the mass, which are fermented by lactic acid bacteria [4]. This happens only with rapid acidification of the feed to a pH of ≤ 4.5 and below [5]. When alfalfa is siloed, even with a dry matter content of 35%, intensive proteolysis occurs [6], accompanied by the accumulation of a large amount of ammonia. Accelerated acidification of the feed does not reduce proteolysis, since the proteolytic enzymes of alfalfa show maximum activity at a pH of about 4.0 [7]. However, this technique is of great importance. It was found [8] that if at a pH of 4.26-4.40, 56 mg of
ammonia is required for its displacement in 100 g of clover silage to the alkaline side by 0.2 units, then at a pH of 6.40-6.60 – only 8 mg [8]. It should also be taken into account that the nutritional value of silage depends not only on the amount of losses during fermentation, but also on the safety of nutrients when it is removed from storage. Dried alfalfa silage is resistant to aerobic spoilage [9]. It is believed [10] that this is due to the presence of secondary metabolites in alfalfa that have bactericidal and fungicidal effects. At the same time, there is evidence that preparations created on the basis of heterofermentative lactobacillus Lactobacillus buchneri improve the aerobic stability of dried alfalfa silage [11].

The aim of the research was to study the peculiarities of the occurrence of aerobic spoilage in a silo of dried alfalfa and to identify the possibility of minimizing it through the use of bacterial preparations.

2. Methods of the research
The experiments were carried out on the basis of the Federal Research Center "V. R. Williams VIC". We used variable alfalfa (Medicago sativa L. notosubsp. varia (Martyn) Arcang), varieties of Taisia, harvested in the budding phase and dried in the prokos to a dry matter content of 37.07%. The crushed mass was siloed in 0.5-liter vessels equipped with devices for accounting for released gases, in the usual way and with the preparations Biotrof (Lactobacillus plantarum 60), Biotrof 2+ (Lactobacillus plantarum 60 and Enterococcus faecium 1-35), Biotal Axfast NS Gold (Lactobacillus plantarum MA 18/5 U, Pediococcus pentosaceus NCIMB 12455, Lactobacillus buchneri NCIMB 40788 and Propionibacterium acidipropionici MA 26/4U), Biotrof 111 (Bacillus subtilis 111. The preparations were administered at the dose recommended by the manufacturers. The biochemical analysis of the feed was carried out after 2 months of storage. At the same time, the feed samples were frozen at a temperature of -25°C and sent to the molecular genetic laboratory of Biotrof LLC, where the number of yeast (Saccharomyces cerevisiae, Hanseniaspora uvarum, Candida stellate, Metschnikowia pulcherrima, Cryptococcus gattii, Debaryomyces hansenii) and micromycetes (Aspergillus sp. and Fusarium) was determined by quantitative PCR methods. The number of microbes was determined after 2 months of feed silage and after its 7-day aeration. During the aerobic stability test, all feed samples were stored in air at room temperature (20-22°C). The beginning of aerobic spoilage of the feed was judged by an increase in its temperature by more than 2.00°C relative to the ambient temperature. The dry matter content in the green mass and silage was determined by drying the canopies at a temperature of 105°C, sugar-according to Bertrand, ammonia - according to Longi, the buffer capacity of the mass - according to Weissbach, pH-using a potentiometer I-500, organic acids (lactic, acetic, butyric, propionic, formic, amber, apple, lemon, wine and oxalic) - by capillary electrophoresis. Statistical processing was carried out using the Student's t-test. The results were considered reliable at P <0.5.

3. Results of the research
The volume of gases released during the silage of alfalfa without additives and with the introduction of the tested preparations, as well as the biochemical parameters of the feed after 60 days of silage and after 7 days of storage in the air are presented in Table 1. From the data presented in Table 1, it follows that all the tested bacterial preparations caused a reduction in the breakdown of nutrients to gaseous products almost twice as compared with conventional silage of dried alfalfa. This is explained by the fact that the rapid acidification of the silage mass with the specified dry matter content to a pH of 4.08-3.97 leads to the suppression of the vital activity of undesirable bacteria during silage. This is also indicated by a reduction in the ammonia content (by 1.5-4.0 times) in the dry matter of the feed. When the dry matter content in the siloed alfalfa is higher than 40.0%, where the activity of undesirable microflora is restrained by low water activity (Ab), bacterial preparations contribute to an increase in the breakdown of nutrients to gaseous products [12]. Butyric acid did not accumulate during the usual silage of dried alfalfa, despite the fact that the pH of the finished silage did not reach the critical value for butyric acid bacteria at a given dry matter content [13].
Table 1. The volume of released gases and biochemical parameters of the silage from dried (37.07% SV) alfalfa, prepared by various methods, after 60 days of silage

| Indicators                     | Silage options          |
|-------------------------------|-------------------------|
|                               | without additives      | with preparations |
|                               |                         | Biotrof | Biotrof 2+ | Biotrof 111 | Biotal | Axfast NS | Gold |
| Volume of released gases, l/kg SV mass | 8,58±0,43                          | 4,38±0,14   | 3,72±0,61   | 4,89±0,19   | 4,46±0,13 |
| Content in the SV, %          |                         |          |            |            |        |          |
| of sugar                      | 3,33±0,08                | 0,79±0,07 | 1,11±0,10 | 1,20±0,07 | 0,37±0,03 |
| of ammonia                    | 0,20±0,007               | 0,07±0,006 | 0,05±0,001 | 0,08±0,007 | 0,13±0,003 |
| of organic acids:             |                         |          |            |            |        |          |
| milk                          | 5,08±0,03                | 12,56±0,05 | 12,93±0,11 | 12,27±0,45 | 12,49±0,31 |
| vinegar                       | 0,75±0,01                | 0,47±0,01 | 0,40±0,02 | 0,56±0,08 | 0,96±0,09 |
| oil                           | 0,00±0,00                | 0,00±0,00 | 0,00±0,00 | 0,00±0,00 | 0,00±0,00 |
| apple                         | 0,57±0,01                | 1,06±0,02 | 1,30±0,02 | 0,95±0,06 | 1,42±0,03 |
| lemon                         | 0,00±0,00                | 0,00±0,00 | 0,00±0,00 | 0,00±0,00 | 0,00±0,00 |
| amber                         | 0,52±0,01                | 0,20±0,021 | 0,13±0,03 | 0,00±0,00 | 0,00±0,00 |
| pH                            | 4,96±0,003               | 4,00±0,003 | 3,97±0,003 | 4,02±0,001 | 4,08±0,006 |

* the difference is significant in relation to silage without additives, P <0,05.

Table 2. Biochemical parameters of dried alfalfa silage after 7 days of aeration

| Indicators | Silage options          |
|------------|-------------------------|
|            | without additives      | with preparations |
|            |                         | Biotrof | Biotrof 2+ | Biotrof 111 | Biotal | Axfast NS | Gold |
| pH         | 4,96±0,003               | 4,00±0,003 | 3,97±0,003 | 4,02±0,001 | 4,08±0,006 |

This confirms the assumption that there are secondary metabolites in alfalfa that have an antimicrobial effect.
Content in dry matter, %

|                  | 0,21±0,007 | 0,09±0,003 | 0,06±0,003 | 0,09±0,003 | 0,15±0,003 |
|------------------|------------|------------|------------|------------|------------|
| of ammonia       |            |            |            |            |            |
| organic acids:    |            |            |            |            |            |
| milk             | 5,22±0,10  | 12,4±0,55  | 12,68±0,89 | 12,88±0,35 | 12,21±0,90 |
| vinerag           | 0,84±0,04  | 0,47±0,02  | 0,36±0,02  | 0,54±0,10  | 0,86±0,01  |
| oil              | 0,17±0,02   | 0,13±0,01  | 0,00±0,00  | 0,19±0,04   | 0,00±0,00  |
| apple            | 0,62±0,05   | 1,15±0,06  | 1,22±0,05  | 1,16±0,06  | 1,40±0,11  |
| lemon            | 0,21±0,02   | 0,00±0,00  | 0,00±0,00  | 0,00±0,00  | 0,00±0,00  |
| amber            | 0,53±0,02   | 0,24±0,03  | 0,21±0,10  | 0,00±0,00  | 0,18±0,02  |
| pH               | 4,79±0,001  | 3,97±0,001  | 3,94±0,003  | 3,99±0,003  | 4,04±0,003  |

* the difference is significant in relation to a similar variant of silage after removal from the vessels, P<0.05.

b no other organic acids were found.

There is reason to believe that this factor also ensured the stability of the silo during its 7-day storage in the air. It follows from the data in Table 2 that at 7-day aeration, the biochemical parameters of the silo did not deteriorate. Moreover, there was even a significant (P <0.05) decrease in pH in silage, both prepared without additives, and with the introduction of the tested drugs. A significant increase in the ammonia content (P <0.05) was observed only during aeration of silage prepared with Biotal Axfast NS Gold. In the aerated silo prepared without additives and with the introduction of Biotorf and Biotorf 111 preparations, the content of butyric acid, which was not detected when opening the silo, also increased significantly (P <-0.05). At the same time, if it accumulated in a silo without additives against the background of a relatively high pH value (4.79), then in a silo with Biotorf and Biotorf 111 preparations – at a pH of 3.97-3.99.
Figure 1. Yeast content in alfalfa silage: a – initial mass, b – silage without additives, c – with Biotrof starter culture, d – with Biotrof-111, e – with Biotrof 2+, f – with Biotal Axfast HC Gold, g – without additives after 7 days of aeration, h – with Biotrof after 7 days of aeration, i – with Biotrof-111 after 7 days of aeration, j – with Biotrof 2+ after 7 days aeration, k – with Biotal Axfast NS Gold after 7 days of aeration, l - significantly compared with the control at the end of fermentation, m - significantly compared with the control when under aerobic exposure.

The figure shows the yeast content at the end of fermentation of the silage and after 7 days of its aeration. The number of almost all studied yeast species (except for Criptococcus gattii) significantly (P <0.05) increased during fermentation of all silage variants. The number of yeast species C. gattii and D. hansenii increased (P <0.05) both in the control and in some variants of the experimental silo, compared with the feed that was not subjected to aerobic exposure. The number of yeasts of the species Saccharomyces cerevisiae, Hanseniaspora uvarum, Candida stellata and Metschnikowia pulcherrima practically did not change during feed aeration. This means that aerobic spoilage of silage is associated with specific types of yeast, and yeast species C. gattii and D. hansenii can play the role of marker organisms, an increase in the number of which indicates the activation of aerobic spoilage of silage. The use of the preparations Biotrof and Biotrof 2+ contributed to a decrease in the number of yeast species D. hansenii during fermentation of silage, and the preparation Biotrof 111 – when the finished silage came into contact with air. The drug Biotrof reduced the number of yeast species C. gattii during fermentation of silage, and Biotrof 111 during its subsequent 7-day aeration. The presence of DNA of a mold fungus of the genus Fusarium sp. no evidence was found in alfalfa silage, which may be related to the resistance of this alfalfa variety to damage by this pathogen [14]. The number of fungi of the genus Aspergillus sp. it increased in comparison with the initial mass in the control silo. The use of the studied drugs led to a decrease in the number of these fungi by 3.7-4.1 times (P <0.05).

4. Discussion
Silage of alfalfa dried to a dry matter content of 37.07% with bacterial preparations leads to an improvement in the safety of feed and an increase in the safety of feeding it to cattle. The latter is due to the biological characteristics of alfalfa and the specific effect of the drugs used. The most pronounced effect associated with a decrease in the content of mold fungi of the genus Aspergillus sp. in the process of aeration of feed, compared with the control, Biotrof was observed in the drug. This is important, since it was previously established [15] that 69% of silage samples from 39 studied variants were infected with A. fumigatus. At the same time, 59.3% of the isolates synthesized the toxin fumitremorgen B, 33.3% - fumitremorgen C, 29.6% - fumigaclavin B, 7.4% - fumigaclavin C and 11.1% of the isolates – gliotoxin. It is known that these mycotoxins, especially gliotoxin, have powerful immunosuppressive, genotoxic, cytotoxic and apoptotic effects. Of particular importance is the revealed ability of the preparations Biotrof 111 and Biotrof 2+ to restrain the development of yeast genera C. gattii and D. hansenii, which are supposed to be the main causative agents of aerobic spoilage. It is also important that yeasts of the genus C. gattii are pathogens that are widely represented in the environment, in particular on plants [16]. The presence of this microbe is often associated with outbreaks of mastitis in cows [17], lung damage, cicatrical tympania, fever in goats [18]. The presence of the microbe C. gattii in the silo was revealed by us for the first time, while the development of yeast of the genus D. hansenii
in a silo of dried alfalfa is quite natural, since they belong to osmotolerant and xelerant organisms and, for this reason, are often found in environments with low water activity [19]. These yeasts are responsible for the spoilage of some foods preserved in brine [20]. The phenomenon of antagonism of lactic acid bacteria and bacteria of the genus Bacillus in the composition of the preparations used is explained by the presence in their genome of a large number of chromosomal loci [16] that determine the synthesis of antimicrobial compounds: organic acids and bacteriocins.

5. Conclusion
The use of the studied bacterial preparations during silage of alfalfa dried to a dry matter content of 37.07% is advisable, since it helps to reduce the breakdown of nutrients to gaseous products and reduce (by 1.5-4.0 times) the formation of ammonia in the feed. It is also important that the use of the studied drugs has a restraining effect on the development of certain types of yeast, including pathogenic ones, as well as fungi of the genus Aspergillus sp., which produce dangerous mycotoxins.

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