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MICROBIOLOGICAL QUALITY OF EXPERIMENTAL SILAGES IN COMBINATION WITH THE ADDITION OF TOPSOIL SOIL LAYER AND ENSILING ADDITIVES

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Abstract

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Fodder crops contamination by the surface layer of soil is important in terms of the occurrence of clostridial spores that can infect silage through the contaminated fodder crops, which is followed by the feeding and occurrence of clostridia in the environment of the stalls resulting in their presence in milk, milk products and finally in the human digestive system. The main objective of the submitted study was to assess the impact of added topsoil layer and ensiling additives on the hygienic quality of the experimental silages. In the model experiment, we used alfalfa from the second cut. In total, the experiment included 9 various treatments in three repetitions. Use was made of the variants of experimental silages P0, P20, P40 (without ensiling additive), B0, B20, B40 (treated with a biological ensiling additive) and CH0, CH20, CH40 (treated with a chemical ensiling additive) always with an addition of a surface layer of soil in a quantity of 0, 20 and 40 g/kg of dry matter. The model silages were assessed after 10-week storage. A representative sample was taken from each experimental variant to perform a microbiological analysis. In the experiment, the counts of clostridia colonies, sporulating microorganisms, anaerobic microorganisms, total number of microorganisms (CPM), lactic acid bacteria, moulds and yeasts were observed. Statistically conclusive difference in the topsoil layer addition was only proved in CPM between variants P20 (1.96E+07 ± 6.21E+06) and P40 (4.97E+07 ± 1.89E+07) and bacteria of the *Enterobacteriaceae* family between variants P0 (2.17E+02 ± 3.32E+01) and P40 (3.64E+01 ± 2.57E+01). A positive effect of ensiling additives on the microorganism growth inhibition was determined.

Keywords: silage, microorganisms, additives, clostridium, soil

INTRODUCTION

Silage as a roughage represents a basis of the beef cattle diet. To prepare good quality silage, only quality fresh or wilted fodder harvested in optimal picking ripeness must be used, while keeping all the technological procedures and principles of the proper production practice (Doležal, 2012). The ensiling principle consists in a fast inactivation of enzyme systems, ensiled fodders, while terminating the microbial activity of the silage matter (Hofírek, 2009). The plant carbohydrates are transformed by the acid-forming, largely lactic fermentation using lactic fermentation bacteria, into lactic acid (Mathies, 2002; Wilkinson, 2005), which rapidly reduce the pH value (Mathies, 2002). Ensiling additives are used to boost the ongoing fermentation process during ensiling. However, they cannot replace it, the requirements for the quality and cleanliness of the ensiling matter or the requirements for sufficient mass stamping

cannot be reduced (Doležal *et al*., 2002). Another product of the fermentation is acetic acid. Both these acids ensure the preserving effect in the silages. However, undesirable acids may also be formed, such as butyric acid, propionic acid, isobutyric acid, n-butyric acid, isovaleric acid, n-valeric acid and capronic acid (Jakobe *et al*., 1987). The created anaerobic conditions have an adverse effect on undesirable microorganisms, whose growth is prevented thanks to the low pH value and lack of oxygen (Mathies, 2002; Šilhánková, 2002). They may reduce the quality (nutrient content, palatability) of the silage; however, they often pose a health risk to animals and potentially also to people as they have a negative effect on the quality of milk and milk products (Wilkinson, 2005).

During fodder harvesting, the topsoil layer can get into silages along with the chopped fodder, bringing undesirable microorganisms therewith. The undesirable microorganisms include mainly clostridia, which are marked as butyric fermentation bacteria, which metabolise hydrocarbons into butyric acid. Clostridia can then affect the digestive system of the livestock along with the contaminated silage, from where they can get to the excrement, and through the contaminated environment, they can also appear in milk. The subsequent processing of contaminated milk into cheese results in typical cheese defects such as late cheese blowing. The vitality of clostridia decreases with the increasing dry matter content over 30%. Hofírek *et al*. (2009) present diseases that can be caused by clostridia. Clostridia in animals result in diseases such as botulism provoked by the toxin of the *Clostridium botulinum* bacteria after oral intake with contaminated feed or water. Another disease is tetanus caused by *Clostridium tetani*. Clostridia penetrate into the organism through deep contaminated wounds. Another disease is blackleg caused by *Clostridium chauvei*. It infects animal organisms on pasture after feeding on the contaminated feed or water. Clostridia in calves may cause inflammation of the umbilicus. Cattle diseases caused by clostridia may result in the death of the animal.

Other undesirable microorganisms include micromycetes (moulds and yeasts). They are indicators of poor storage conditions, low standard of hygiene and they cause health and nutrition problems. Yeasts are considered to be the main cause of aerobic instability of silage (zeman, 2006). The effects of acetic acid and propylene glycol during fermentation strongly inhibit moulds and yeasts (Mathies, 2002). Growing moulds produce toxic substances of non-protein nature – mycotoxins (Osweiller, 2000). For nutritious reasons, it is important to extend the storage period without the occurrence of spontaneous, undesirable, mechanical, biochemical and microbial changes in the feed (Havlíček *et al*., 2008).

Bacteria in the *Enterobacteriaceae* family are a part of the livestock as well as human intestinal micro flora and they occur in the natural environment (Greif *et al*., 2006) and indicate faecal contamination of silage.

The main objective of the study was to validate the hypothesis whether the addition of ensiling additives into the model alfalfa silage intentionally contaminated with the topsoil layer may suppress the growth of undesirable microorganisms.

MATERIALS AND METHODS

The experimental material was obtained from the Research Institute of Fodder Crops in Troubsko The model experiment used alfalfa *(Medicago sativa, var. Pálava)*, which was obtained from the second cut from the experimental plots. Experimental plants were sampled taking into account actual agronomical conditions. Alfalfa was harvested at the bud stage and shortly wilted (6 hours) to a final dry matter content of 31%. This was followed by a model two-factor experiment, with the first monitored factor being the addition of topsoil layer at a dose of 0, 20 and 40 g/kg of dry matter (Tab. I). The doses of added surface soil were set with respect to the expected ash content in the original matter (around 8%/kg of dry matter) and the intended ash content in the contaminated silages exceeding 10%, which is in practical terms considered as the limit ash matter content value. For contamination, use was made of the surface layer of soil obtained from the experimental plots (max. depth used for the extraction was up to 1 cm). The second factor was the addition of biological and chemical ensiling additives and their influence on the silage micro flora. A control treatment was used in the experiment (with the addition of an equivalent volume of drinking water to keep the same dry matter content), biological additive (at a dose of 20 g/t) and chemical agent (2 l/t). Composition of the biological inoculants: *Enterococcus faecium, Lactococcus lactis,* EC xylanase enzyme. The chemical agent was based on a mixture of organic acids and salts (formic acid 43%, propionic acid 10%, butyric acid 2%, ammonium formate 30%). During the experiment, altogether nine silage variants were prepared in three repetitions according to the scheme shown in Tab. I.

Such a treated matter, weight of 6 kg, was stamped using a pneumatic press into special ensiling vessels the design of which enables hermetic closing and a constant load of the ensiling matter. Each treatment was provided in three repetitions. The model silages were stored for a period of 10 weeks in a laboratory at an average laboratory temperature of 24–26 °C. After opening the ensiling vessels, the vessels were weighed (to record weight loss during fermentation) and a sample was taken from each treatment to determine the dry matter content and to perform a microbiological analysis.

Twenty grams of samples were shaken for 15 minutes in a shaking apparatus together with 180 ml of physiological solution. Then,

I: *Scheme of the experiment*

P0 – control (without treatment)	B0 – with the addition of biological additive	CH0 – with the addition of chemical additive
P20 – control with soil addition 20 g/kg dry matter	B20 – with the addition of biological additive and soil – 20 g/kg dry matter	CH20 – with the addition of chemical additive and soil – 20 g/kg dry matter
P40 – control with soil addition 40 g/kg dry matter	B40 – with the addition of biological additive and soil – 40 g/kg dry matter	CH40 – with the addition of chemical additive and soil – 40 g/kg dry matter

a series of decimal dilutions was prepared and 1 ml of the relevant dilution was inoculated into Petri dishes with the addition of a live medium.

To determine the total count of microorganism (CPM), PCA agar (Biokar Diagnostics, France) was used as a cultivation medium; incubation was taking place at 30 °C for a period of 72 hours. To determine the count of lactic acid fermentation bacteria (BMK), use was made of MRS agar (Biokar Diagnostics, France); incubation was taking place for 72 hrs at 30 °C. To determine yeast and moulds, use was made of Chloramphenicol Glucose Agar (Biokar Diagnostics, France); incubation was taking place for 120 hrs at 25 °C. To determine sulphide-reducing clostridia (*Clostridium perfringens*), use was made of TSN agar (Biokar Diagnostics, France); incubation was taking place in anaerobic conditions at 46 °C for a period of 24 h. For the determination of the count of thermo-resistant anaerobic (sporulating) microorganisms, use was made of PCA agar (Biokar Diagnostics, France) over 48–72 hrs at 30 °C, after pasteurisation at 85 °C, 10 min. After the incubation, the developed colonies were determined from the Petri dishes and the results of the analysis were expressed as CFU/gram of silage.

The results were processed statistically using the method of variation analysis and the differences between the individual groups were assessed by Scheffe's test according to Snedecor and Cochran (2012). Differences between all treatments and differences between treatments with the addition of topsoil layer, biological and chemical ensiling additives were observed. The statistically significant difference is shown in the table using indices. Treatments with the same indices are not statistically significant. Data is presented in the text as mean ± standard deviation.

RESULTS AND DISCUSSION

The objective of the experiment was to determine the effect of an addition of topsoil layer and biological and chemical ensiling additives on the occurrence of microorganisms in the experimental alfalfa silage. The silage was monitored with respect to the occurrence of bacteria of the *Enterobacteriaceae* family, clostridia, total count of microorganisms (CPM), thermo-resistant (sporulating) microorganisms, anaerobic microorganisms, lactic acid fermentation bacteria (BMK), moulds and yeasts.

In the experimental samples of all treatments, the bacteria of the *Enterobacteriaceae* family occurred in very small counts. According to Kung (2009), the fermentation process is started by microorganisms in the *Enterobacteriaceae* family that ferment the present carbohydrates into acetic acid. These bacteria are then inhibited by the activity of the lactic acid fermentation bacteria. The comparison of silages with the addition of the topsoil layer showed a statistically significant difference ($P < 0.05$) between ensiling variants P0 and P40 (Tab. II). The correct fermentation process in the silages resulted in a reduction of the *Enterobacteriaceae* bacteria family count. Following the treatment of the experimental silages with chemical and biological additives, a statistically significant drop in the number of *Enterobacteriaceae* bacteria family count compared to the control group was determined. As regards variant P40, there was no significant difference in the control group and compared to the applied preservative.

With respect to the experimental silages it was determined that the addition of topsoil layer in the amount of 0, 20, 40 g/kg of dry matter did not result in a statistically significant difference in the occurrence of anaerobic microorganisms (Tab. III). With the addition of ensiling additives, count of anaerobic microorganisms in the experimental alfalfa silage was reduced.

II: *The content of Enterobacteriaceae in the experimental silages in CFU/g*

Microorganism	Effect of	Variant	Average ± st.deviation	Indices
Enterobacteriaceae family	soil	P0	2.17E+02 ± 3.32E+01	b
		P20	1.15E+02 ± 7.12E+01	ab
		P40	3.64E+01 ± 2.57E+01	a
	preservative	K	3.05E+02 ± 9.12E+01	b
		B	2.98E+01 ± 9.92E+00	a
		CH	3.28E+01 ± 2.90E+01	a

Different letters in the same column indicate significant differences at $P < 0.05$ (ANOVA)

III: *The content of anaerobic microorganisms the experimental silages in CFU/g*

Microorganism	Effect of	Variant	Average ± st. deviation	Indices
Anaerobic microorganisms	soil	P0	1.82E+04 ± 1.19E+04	a
		P20	5.31E+03 ± 1.22E+03	a
		P40	5.24E+03 ± 6.03E+02	a
	preservative	K	2.86E+04 ± 1.35E+04	b
		B	1.18E+02 ± 1.11E+02	a
		CH	8.84E+01 ± 4.24E+01	a

Different letters in the same column indicate significant differences at $P < 0.05$ (ANOVA)

IV: *The content of sporulating microorganisms in the experimental silages in CFU/g*

Microorganism	Effect of	Variant	Average ± st.deviation	Indices
Thermo-resistant anaerobic (sporulating) microorganisms	soil	P0	2.97E+02 ± 1.29E+02	a
		P20	4.54E+02 ± 2.85E+02	a
		P40	2.77E+02 ± 1.11E+02	a
	preservative	K	7.13E+02 ± 3.85E+02	b
		B	1.64E+02 ± 1.16E+02	a
		CH	1.52E+02 ± 2.39E+01	a

Different letters in the same column indicate significant differences at $P < 0.05$ (ANOVA)

Counts of anaerobic microorganisms in silages treated with the biological or chemical ensiling additives with the addition of topsoil layer did not change significantly. Thus, it may be stated that the addition of ensiling additives to the experimental alfalfa silages resulted in a reduced count of anaerobic microorganisms.

The highest counts of anaerobic microorganisms in untreated silages with the addition of topsoil layer and even without the addition of the topsoil layer reached the maximum values of 10^4 CFU/g and those treated with chemical or biological ensiling additives totalled 10^1 CFU/g–10^2 CFU/g.

According to Bolsen (1992), the occurrence of clostridia in silages with moisture content below 65% is rather unique. The dry solid content of the experimental alfalfa silage was 31%. Doležal (2012) claims that fast wilting of the experimental material to dry matter content over 35% is the most efficient measure against the occurrence of clostridia in silage. Loučka *et al.* (1997) informed that clostridia stop proliferating at a water activity value (a_w) of 0.94.

Sulphide-reducing clostridia were not detected in the samples of experimental silages.

The highest counts of thermo-resistant anaerobic (sporulating) microorganisms (are mainly represented by the genus *Clostridum* or rarely by the genus *Bacillus*) in silages with the addition of topsoil layer and after the treatment with ensiling additives reached the values of 10^2 CFU/g (Tab. IV). The assessment of the effect of the addition of topsoil layer in various quantities did not determine any statistically significant difference. The count of sporulating microorganisms was the highest in the control group and following the treatment of the experimental silages with biological and chemical ensiling additive there was a statistically significant reduction in the sporulating microorganism counts.

ZEMAN *et al.* (2006) maintain that the highest of spore clostridia count should be approx. up to $5*10^3$ CFU/g. This theory is also confirmed by Doležal (2012), which corresponds to values up to 5,000 clostridia spores in 1g of silage. In the experiment, we can observe a reduced clostridia count in using ensiling additives.

In the experimental silages with the addition of topsoil layer we determined the highest total microorganism counts in variants P40 (4.97E+07 ± 1.89E+07), but this difference was not statistically significant (Tab. V). This group of microorganisms was examined to determine the general level of microbial presence in the silages. The total count of microorganisms is strongly affected by the counts of lactic acid fermentation bacteria, which participate in the fermentation process in the silages. A statistically significant difference was demonstrated between variants P20 and P40. The addition of ensiling additives resulted in the reduction of the total microorganism count in the experimental silages. The CPM reduction in the experiment was most efficient with using the chemical ensiling additive. The achieved results correspond with the findings of Bolsen (1992), Wilkinson (2005).

The lactic acid fermentation bacteria did not show any statistically significant effect of the addition of topsoil layer ($P < 0.05$). Statistically significant differences (Tab. VI) were determined between the control variant (3.47E+07 ± 1.12E+07) and the variant treated with the chemical ensiling additive (5.52E+06 ± 5.04E+06) and between the treatment with the biological ensiling additive

V: *The total number of microorganisms in the experimental silages in CFU/g*

Microorganism	Effect of	Variant	Average ± st.deviation	Indices
Total number of microorganisms	soil	P0	2.95E+07 ± 1.14E+07	ab
		P20	1.96E+07 ± 6.21E+06	a
		P40	4.97E+07 ± 1.89E+07	b
	preservative	K	5.56E+07 ± 1.77E+07	a
		B	3.53E+07 ± 1.22E+07	a
		CH	7.90E+06 ± 6.73E+06	b

Different letters in the same column indicate significant differences at $P < 0.05$ (ANOVA)

VI: *The content of LAB microorganisms in the experimental silages in CFU/g*

Microorganism	Effect of	Variant	Average ± st.deviation	Indices
Lactic acid fermentation bacteria	soil	P0	1.77E+07 ± 8.40E+06	a
		P20	1.77E+07 ± 5.90E+06	a
		P40	3.71E+07 ± 1.82E+07	a
	preservative	K	3.47E+07 ± 1.12E+07	a
		B	3.22E+07 ± 1.62E+07	a
		CH	5.52E+06 ± 5.04E+06	b

Different letters in the same column indicate significant differences at $P < 0.05$ (ANOVA)

VII: *The content of yeasts in the experimental silages in CFU/g*

Microorganism	Effect of	Variant	Average ± st.deviation	Indices
Yeasts	soil	P0	2.33E+02 ± 1.68E+02	a
		P20	1.31E+02 ± 1.01E+02	a
		P40	3.38E+01 ± 1.24E+01	a
	preservative	K	3.75E+02 ± 2.78E+02	b
		B	0.00E+00 ± 0.00E+00	a
		CH	2.27E+01 ± 3.71E+00	a

Different letters in the same column indicate significant differences at $P < 0.05$ (ANOVA)

(3.22E+07 ± 1.62E+07) and chemical additive (5.52E+06 ± 5.04E+06). After the treatment of experimental silages using the chemical additive, the lactic acid fermentation bacteria count was reduced by one order of magnitude. The highest lactic acid fermentation bacteria reached the values of 10^7 CFU/g. Doležal *et al.* (2006) state that lactic acid fermentation bacteria in the silage material compete with yeast and the general rule is that the higher the BMK content, the easier the primary fermentation process.

The yeast count was decreasing with the growing quantity of the added topsoil layer; however, no statistically significant difference was determined (Tab. VII). In evaluating the quality of microbiological analysis, no statistically significant influence of ensiling additives on the yeast occurrence was determined. This goes against the findings of Filya (2003) who detected an inhibition of yeast formation after the treatment of silage using the biological ensiling additive and Kleinschmit *et al.* (2005) whose experiments show a positive effect of a biological ensiling additive (based on *Lactobacillus buchneri*) on the yeast count reduction in silages. As regards the silages treated with biological or chemical ensiling additives and untreated silages, the yeast count was significantly lower as compared with the control treatment. Lád *et al.*, (2008) confirm the effect of ensiling inoculants on the fermentation process quality. The applied ensiling additives did not feature any statistically significant differences in the yeast count. Currently, no permissible limits of the mould and yeast count in preserved fodder are imposed. However, according to Zeman *et al.* (2006), the yeast count limits should reach a maximum concentration of 10^4 CFU/g. The highest yeast count in all the experimental treatments reached a value of 10^2 CFU/g. Thus, it may be concluded that the yeast counts in the experimental silages did not exceed the values presented by Zeman *et al.* (2006). The silages become warmed up during the activity of yeasts and their concentration increases to above 10^7 or 10^8 CFU/g. This was also confirmed by the study of Bolsen (1992).

The experimental silage samples were not visibly mould-infected. A statistically significant difference (Tab. VIII) in the mould count with the addition of topsoil layer was not demonstrated. The mould counts reached the maximum values up to 10^2 CFU/g in all experimental treatments. Mudřík

VIII: *The content of moulds in the experimental silages in CFU/g*

Microorganism	Effect of	Variant	Average ± st.deviation	Indices
Moulds	soil	P0	3.18E+01 ± 1.67E+01	a
		P20	7.32E+01 ± 4.02E+01	a
		P40	1.31E+01 ± 8.44E+00	a
	preservative	K	1.00E+02 ± 5.47E+01	b
		B	6.06E+00 ± 3.32E+00	a
		CH	1.21E+01 ± 7.28E+00	a

Different letters in the same column indicate significant differences at $P < 0.05$ (ANOVA)

et al. (2006) along with Zeman (2006) consider a limit up 10^5 CFU/g as suitable for moulds, and the experimental alfalfa silages met the limit. This theory is also confirmed by Doležal (2012). The experimental silages comply with the hygienic quality of silage. If the mould concentration is higher than 10^6 or 10^7 CFU/g, the fodder is considered spoilt and non-feedable. Wilkinson (2005) assumes that toxinogenic mould species are expected to be found at a higher occurrence of moulds.

A statistically significant difference in moulds ($P < 0.05$) was demonstrated between variant K (control) and silages treated with the chemical and biological ensiling additives (B, CH). Similarly, WYSS (2002) proved the effect of the chemical preservative based on benzoic acid- and propionic acid salts on inhibiting the yeasts and moulds development during the fermentation process of brewer's grains ensiling.

In general, chemical ensiling additives based on organic acids and their salts have considerable anti-fungal and antibacterial effects as confirmed by other authors, too (Lättemäe, Lingvall, 1996; Wilkinson, 2005). Likewise, Knický, Spörndly (2010) described increased aerobic stability and reduced loss after the use of chemical ensiling additives.

CONCLUSIONS

The effect of the addition of topsoil layer showed a statistically significant difference ($P < 0.05$) in the *Enterobacteriaceae* bacteria family and in the total microorganism count. As regards yeasts with the addition of topsoil layer, their count was gradually reduced; however, with no statistically significant difference. No effect of the addition of topsoil layer on clostridia, sporulating microorganisms, anaerobic microorganisms, lactic acid fermentation bacteria, yeasts and moulds was detected. It may be concluded that a positive effect of the addition of biological and chemical ensiling additives into experimental silages was demonstrated, resulting in a reduced count of the *Enterobacteriaceae* family bacteria, sporulating microorganisms, anaerobic microorganisms, yeasts, and in an improved quality of the fermentation process. Following the chemical ensiling additive treatment, the bacteria of lactic fermentation showed a count reduction by an order of magnitude. The experiment corroborated the hypothesis that the use of ensiling additives results in a suppressed growth of undesirable microorganisms and the selected contamination of experimental alfalfa silages by the topsoil layer results in the reduction of some microorganisms only.

SUMMARY

The main objective of the experiment was to assess the effect of the addition of topsoil layer and ensiling additives on the occurrence of microorganisms in alfalfa silage by the microbiological analysis. The experiment included alfalfa, harvested in the second cut, at the bud stage and following a short wilting to a dry matter content of 31%. A two-factor experiment was conducted with the first monitored factor being the effect of the addition of topsoil layer (extraction from the experimental plots at a max. depth of 1 cm) in doses of 0, 20 and 40 g/kg of dry matter. The doses of added surface soil were set with respect to the expected ash content in the original matter (around 8%/kg of dry matter) and the intended ash content in the contaminated silages exceeding 10%, which is in practical terms considered as the limit ash matter content value. The second factor was the addition of biological and chemical ensiling additives and their influence on the silage microbiological analysis. The experiment used variants of experimental silages P0, P20, P40 (without ensiling additives), B0, B20, B40 (treated with a biological ensiling additive) and CH0, CH20, CH40 (treated with a chemical ensiling additive) always with the addition of a topsoil layer at a quantity of 0, 20 and 40 g/kg of dry matter. Such a treated material with a weight of 6 kg was stamped into silage vessels. The experiment included a total of 9 variants in three repetitions. The experimental silages were stored for 10 weeks at a temperature of 24–26 °C. Following the fermentation, a representative sample was taken from each experimental treatment for the purpose of microbiological analysis. The experiment monitored the counts of sulphide-reducing clostridia, thermo-resistant anaerobic (sporulating)

microorganisms, total microorganism count, anaerobic microorganisms, lactic acid fermentation bacteria, moulds and yeast. The results of the microbiological analysis (CFU/g) were then statistically processed in Statistics 10 (ANOVA), using Scheffe's test and the differences between all treatments were evaluated. The effect of the addition of upper soil layer was a statistically significant difference ($P < 0.05$) in bacteria of the *Enterobacteriaceae* family between variants P0 and P40 and in the total microorganism count between variants P20 and P40. As regards yeasts with the addition of the upper soil layer, their count was gradually decreasing; however, there was no statistically significant difference. No effect of the upper soil layer on clostridia, sporulating microorganism, anaerobic microorganisms, BMK, yeast and moulds was observed. A positive effect of the addition of biological as well as chemical ensiling additive in the experimental silages was demonstrated, which resulted in the reduced count of the *Enterobacteriaceae* family bacteria, sporulating microorganisms, anaerobic microorganisms, moulds, yeasts and in the improvement of the fermentation process quality.

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