

## The effects of red grape pomace inclusion in grower diet on amino acid digestibility, intestinal microflora, and sera and liver antioxidant activity in broilers

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**Abstract:** The objective of this study was to evaluate the effect of dietary red grape pomace (RGP) on amino acid digestibility (AIAAD), intestinal microflora, and antioxidant activity in the sera and liver. Sixty-six broilers were housed in battery cages. They were allocated to six cages corresponding to two dietary treatments with three replicates of each treatment. The treatments were administered from 10 to 36 days and included a control diet and a diet containing 1.5% RGP dry powder with the other components remaining unchanged, compared to the control. The RGP had a significant ( $P < 0.05$ ) positive effect on the apparent metabolizable energy. The majority of coefficients of AIAAD were higher in the RGP. The ileal digestibility of threonine, serine, proline, and glycine was significantly ( $P < 0.05$ ) higher in RGP. RGP had a positive effect ( $P < 0.05$ ) on the content of *Lactobacillus* in the lower part of the ileum. The blood sera of broilers fed RGP had significantly ( $P < 0.05$ ) lower 2,2-diphenyl-1-picrylhydrazyl (DPPH) and uric acid content. The RGP at a concentration of 15 g/kg in the grower diet did not affect the growth of broilers, and RGP is suitable for broiler diets to improve gastrointestinal tract functionality.

**Key words:** Red grape pomace, digestibility, threonine, *Lactobacillus*

### 1. Introduction

Grapes (*Vitis* sp., Vitaceae) are one of the most commonly consumed fruits worldwide. The quantity of grapes processed worldwide is approximately 50.0 million tons (Mt) annually, which represents approximately 5.0–9.0 Mt of grape pomace (GP) production (1). There are many studies investigating how to utilize this byproduct, which is wine industry waste in many cases. Pomace consists of skins, stalks, and seeds. Red GP is rich in phenolic compounds with high antioxidant activity (2). Lafka et al. (3) reported higher antioxidant activity in ethanol extract from winery waste than in other solvent extracts, synthetic food antioxidants, butylated hydroxytoluene (BHT), ascorbyl palmitate, and vitamin E, but they did not find a correlation between antioxidant activity and total phenol content. The seeds (38%–52%, on a dry matter basis) also contain high levels of unsaturated fatty acids, mainly linoleic acid (4,5). High levels of proanthocyanidin, which is a type of flavonoid known for its high antioxidant properties, are responsible for the greatest contribution to antioxidant activity (6). There have been a number of

studies regarding the use of GP in human nutrition, e.g., the use of pomace as a source of dietary fiber in bread (7) or to improve the nutrition profile of frankfurters (8). There have also been studies regarding the use of GP as animal feed. Abarghuei et al. (9) observed the effect of GP on the ruminal parameters of sheep, and Molina-Alcaide et al. (10) evaluated wine industry byproducts as feed for ruminants. Alipour and Rouzbehan (11) tested the effects of tannins from GP on the intestinal digestibility of soybean meal. The beneficial effects of tannins are associated with their capacity to increase digestive utilization of dietary protein in ruminants by slowing down the microbial degradation of proteins. Brenes et al. (12) tested grape seed extract in broiler diets, and Goni et al. (13) used GP in combination with vitamin E and found no effect of the extract or GP/vitamin E on ileal protein digestibility, amino acid digestibility, or growth performance in broilers (at 42 days old).

Rotava et al. (14) reported high antibacterial activity of defatted grape seed extract against *Staphylococcus aureus* and *Escherichia coli* strains in broilers, and they

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also reported that the antioxidant activity of the extract is comparable to that of ascorbic acid. Viveros et al. (15) also concluded that dietary polyphenol-rich grape products modify gut morphology and intestinal microflora and increase the biodiversity of intestinal bacteria in broilers.

GP as a byproduct of wine making may have a role in animal nutrition due to the phenolic compound content of the skin and seeds. GP is used mainly in ruminant diets without any treatment, but it is necessary to dry the pomace for poultry nutrition. There have been few studies on the use of GP in poultry nutrition, but authors used extract of grape seeds (12) or GP in combination with vitamin E (13). There are almost no studies using both skin and seeds together in dried GP. Due to the compounds contained in red GP, it may have antimicrobial potential and, consequently, may positively influence growth.

The objective of this study was to evaluate the effect of dietary red GP (both skin and seeds) on amino acid digestibility, apparent metabolizable energy, apparent metabolizable nitrogen, intestinal microflora, and antioxidant activity in the sera and liver of broilers.

## 2. Materials and methods

### 2.1. Grape pomace

Red GP (RGP) with skins and pulp was provided by a small-scale wine producer, and the skin, seeds, and stalk were all dried together at 60 °C. After drying, dry matter content was 93.7%, and the crude protein content was 12.8%. The phenolic compound content in RGP is shown in Table 1.

### 2.2. Bird housing and experimental design

A total of sixty-six 1-day-old female broilers (Ross 308) were housed in battery cages in two tiers with a wire floor in an environmentally controlled room. Heating was provided by electrical brooders; the initial temperature was 32 °C and linearly decreased to 20 °C at 28 days of age. From the beginning to the end of the study the broilers were provided with 1 h of darkness following a period of 23 h of

**Table 1.** Content of phenolic compounds in grape pomace.

Phenolic compounds	µg/g DM RGP
Gallic acid	22.2
Syringic acid	16.7
3,4-Dihydroxybenzaldehyde	11.4
Protocatechuic acid	11.1
Vanillic acid	10.1
P-coumaric acid	3.5
Caffeic acid	1.7

light. Each cage was equipped with an outside single feeder with hand feeding and a nipple line with three nipples per cage. Up to the age of 7 days, the cages contained extra round drinkers and feed troughs with grids. Access to feed and water was provided ad libitum. Up to the age of 10 days, there were 22 birds per cage, they were housed in three cages only in the top tier, and they were fed the same starter diet. After 10 days of age, the birds were randomly allocated to six cages in both tiers corresponding to two dietary treatments with three replicates of each treatment for 36 days. Each group comprised 3 cages containing 11 broilers each. The ingredients and nutrient composition of the diets are shown in Table 2. The dietary treatments included a control (C) diet and a diet containing 1.5% red grape pomace (RGP) dry powder, with the other components remaining the same as in the control diet.

### 2.3. Live weight

The broilers were individually weighed at 10, 20, 30, and 35 days of age. Feed intake per cage was determined from 0 to 10 days and from 10 to 35 days. The feed:gain ratio per cage was derived from the total live weight at 35 days of

**Table 2.** Diet formulations and calculated analyses.

Ingredient (%)	Starter	Grower	
		C	RGP
Wheat	30.0	43.0	41.5
Corn	30.0		22.0
Soybean meal	32.0		27.0
Rapeseed oil	4.0		4.0
Vit/mineral premix*	4.0		4.0
Red grape pomace	0.0	0.0	1.5
Determined analysis			
AME <sub>N</sub> (MJ/kg)**	12.4		12.4
Crude protein (N*6.25, g/kg)	212.1		198.9
Methionine (g/kg)	4.65		4.45
Lysine (g/kg)	12.7		11.5
Calcium (g/kg)	9.96		9.90

\*Supplied per kilogram diet: P 3.5 g/kg; Mg 0.6 g/kg; Na 1.6 g/kg; Zn 80 mg/kg; Mn 100 mg/kg; Fe 80 mg/kg; Cu 8 mg/kg; I 1 mg/kg; Se 0.2 mg/kg; vit. A 12,000 IU; vit. D3 3000 IU; vit. E 50 mg/kg; vit. K3 3 mg/kg; vit. B1 2 mg/kg; vit. B2 6 mg/kg; vit. B6 4 mg/kg; vit. B12 0.02 mg/kg; biotin 0.15 mg/kg; niacin 60 mg/kg; folic acid 1.5 mg/kg; CalPan 14 mg/kg; choline 0.4 g/kg.

\*\*Calculated analysis. Diets were supplied with Cr<sub>2</sub>O<sub>3</sub> by premix (1.5 g/kg). C – control group without red grape pomace, RGP – group with 15 g/kg of red grape pomace.

age and total cage feed intake. The intake up to 10 days of age was calculated to be the same for all birds.

#### 2.4. Amino acid digestibility

At 36 days of age, 24 birds from each group were killed by decapitation after stunning, according to the Czech law (246/1992) to protect animals against cruelty. The small intestine was immediately exposed, and the contents of the lower third of the ileum (3 cm from the ileocecal junction) were collected by gently pressing into petri dishes. The ileal contents of four random broilers from one cage were combined into one dish with six repetitions per group. Samples were stored at  $-20\text{ }^{\circ}\text{C}$ , and the samples were lyophilized, ground, and analyzed. The diets contained chromic oxide ( $\text{Cr}_2\text{O}_3$ ) as an undigested marker and indicator of nutrient digestibility (16).

#### 2.5. Analytical methodology

Diets and excreta were analyzed for nitrogen (Kjeldahl method, AOAC (17) ID 954.01), CP ( $\text{N} \times 6.25$ ), dry matter for 24 h in a forced air oven ( $103\text{ }^{\circ}\text{C}$ ), lipids (Soxhlet extraction, (17) ID 920.39), energy (calorimetric), and  $\text{Cr}_2\text{O}_3$ . After hydrolysis, samples of digesta and diets were analyzed for amino acids by chromatographic analysis using the AAA 400 analyzer (f Ingos). High-performance liquid chromatography (HPLC) with electrochemical and UV-VIS detection were used for phenolic compound determination.

#### 2.6. Microbiological analysis

At 36 days of age, six broilers from each group were killed by cervical dislocation, and their intestinal tracts were removed. Fresh digesta (5 g) from the lower ileum (3 cm from the ileocecal junction) was immediately used for microbiological cultivation, and the number of colony forming units (CFUs) was counted. The digesta was placed into sterile beakers, diluted with distilled water, and homogenized for 3 min in a Stomacher homogenizer. Each digesta homogenate was serially diluted from  $10^{-7}$  to  $10^{-9}$ . One milliliter of the serial dilution was inoculated into sterile petri dishes and sealed with an appropriate medium. The *Lactobacillus* spp. colony count was determined using MRS agar (Biokar Diagnostic, France) after incubation in an anaerobic chamber at  $37\text{ }^{\circ}\text{C}$  for 72 h. *Escherichia coli* colonies were counted on Rapid *E. coli* agar (BIO-RAD) after aerobic incubation at  $37\text{ }^{\circ}\text{C}$  for 24 h. After cultivation in petri dishes, the accrued colonies were counted, and the result was expressed in CFU/g of digesta.

#### 2.7. Biochemical characteristics of blood sera and liver supernatant

The metallothionein (MT) content in the blood sera and liver supernatant was analyzed by an adsorptive transmission technique with differential pulse voltammetry (18). The reduced glutathione (GSH) and oxidized glutathione (GSSG) content was analyzed by

HPLC-ED in both the blood sera and liver supernatant (18). The chromatographic system consisted of two solvent delivery pumps, Zorbax eclipse, and a CoulArray electrochemical detector. The electrochemical detector included three flow cells. Both the detector and the reaction coil/column were thermostated. The free radical, diphenylpicrylhydrazyl (DPPH), and uric acid content of the blood sera was measured using the BS-400 automatic analyzer (Mindray, China) at wavelengths of 450, 505, and 546 nm, respectively (19).

#### 2.8. Statistical analysis

Live weight data were analyzed by t-test, and the feed:gain ratio, digestibility coefficients, blood characteristics, liver characteristics, and microorganism CFUs were analyzed by the nonparametric Mann-Whitney U-test using the Unistat 5.1 software package (Unistat Ltd., England). Statistical significance was determined at  $P < 0.05$ .

### 3. Results

Table 3 shows the growth performance and feed:gain ratio in broilers up to 35 days of age. There was no significant difference between groups at 10 days of age when the experimental period started. The diet containing RGP did not have a significant effect on growth or the feed:gain ratio. At 35 days of age, there was a difference of 16 g in the average weight between groups.

The characteristics of nutrient digestibility and metabolizable energy are shown in Table 4. The diet containing RGP had a significant ( $P < 0.05$ ) positive effect on the apparent metabolizable energy (AME). The apparent metabolizable nitrogen (AMN) was also higher

**Table 3.** Live weight of broilers and feed:gain ratio.

	C	RGP
n	33	33
	Mean $\pm$ SE	Mean $\pm$ SE
Live weight (g)		
10 days	215.1 $\pm$ 5.97	226.5 $\pm$ 6.58
20 days	675.8 $\pm$ 21.1	690.4 $\pm$ 23.7
30 days	1323.8 $\pm$ 52.7	1349.7 $\pm$ 49.9
35 days	1770.0 $\pm$ 50.6	1754.5 $\pm$ 64.0
Feed:gain ratio (kg/kg)		
0–35 days	1.63 $\pm$ 0.014	1.70 $\pm$ 0.034

SE – standard error.

There is no significant difference between groups ( $P > 0.05$ ).

C – control group without red grape pomace, RGP – group with 15 g/kg red grape pomace.

**Table 4.** Ileal digestibility coefficients of amino acids and total tract digestibility coefficients of fat.

n	C	RGP
	24	24
	Mean ± SE	Mean ± SE
Apparent ileal amino acid digestibility		
Lysine	0.888 ± 0.0140 <sup>a</sup>	0.916 ± 0.0032 <sup>a</sup>
Methionine	0.948 ± 0.0165 <sup>a</sup>	0.912 ± 0.0203 <sup>a</sup>
Threonine	0.764 ± 0.0393 <sup>a</sup>	0.875 ± 0.0126 <sup>b</sup>
Arginine	0.890 ± 0.0171 <sup>a</sup>	0.920 ± 0.0042 <sup>a</sup>
Histidine	0.858 ± 0.0140 <sup>a</sup>	0.869 ± 0.0128 <sup>a</sup>
Isoleucine	0.830 ± 0.0284 <sup>a</sup>	0.874 ± 0.0095 <sup>a</sup>
Leucine	0.856 ± 0.0206 <sup>a</sup>	0.881 ± 0.0066 <sup>a</sup>
Valine	0.854 ± 0.0264 <sup>a</sup>	0.888 ± 0.0068 <sup>a</sup>
Phenylalanine	0.871 ± 0.0172 <sup>a</sup>	0.899 ± 0.0095 <sup>a</sup>
Tyrosine	0.823 ± 0.0147 <sup>a</sup>	0.769 ± 0.0328 <sup>a</sup>
Cysteine	0.848 ± 0.0392 <sup>a</sup>	0.914 ± 0.0183 <sup>a</sup>
Aspartic acid	0.819 ± 0.0133 <sup>a</sup>	0.841 ± 0.0105 <sup>a</sup>
Serine	0.818 ± 0.0237 <sup>a</sup>	0.889 ± 0.0140 <sup>b</sup>
Glutamic acid	0.901 ± 0.0065 <sup>a</sup>	0.907 ± 0.0103 <sup>a</sup>
Proline	0.899 ± 0.0069 <sup>a</sup>	0.924 ± 0.0038 <sup>b</sup>
Glycine	0.745 ± 0.0240 <sup>a</sup>	0.810 ± 0.0129 <sup>b</sup>
Alanine	0.831 ± 0.0152 <sup>a</sup>	0.847 ± 0.0070 <sup>a</sup>
Total tract apparent digestibility of fat		
Fat	0.899 ± 0.0040 <sup>a</sup>	0.883 ± 0.0066 <sup>a</sup>
Apparent metabolizable nutrients		
Nitrogen	0.594 ± 0.0180 <sup>a</sup>	0.637 ± 0.0153 <sup>a</sup>
Energy	0.780 ± 0.0042 <sup>a</sup>	0.796 ± 0.0024 <sup>b</sup>

SE – standard error.

a, b – means of the same row designated by different letters are significantly different ( $P < 0.05$ ).

C – control group without red grape pomace, RGP – group with 15 g/kg red grape pomace.

in the RGP group, but the difference of 0.043 was not significant. The majority of the coefficients of apparent ileal amino acid digestibility were higher in the RGP group compared to the control group. The ileal digestibility of threonine, serine, proline, and glycine was significantly ( $P < 0.05$ ) higher in the RGP broilers compared to the control broilers. RGP did not have a significant effect on

the total tract apparent fat digestibility. The bacteriological data are shown in Table 5. The diet containing RGP had a positive effect ( $P < 0.05$ ) on the content of *Lactobacillus* in the lower part of the ileum. There was not a significant difference in the number of *Escherichia coli* CFUs between the two groups.

The antioxidative potential was evaluated in the liver supernatant by measuring the metallothionein and reduced glutathione content, and there was not a significant difference between the C and RGP groups with regard to these characteristics (Table 6).

There was not a significant difference between the C and RGP groups when comparing the MT, GSH, GSSG, and free radical content in the blood sera. In the blood sera of broilers fed RGP, however, there was a significantly ( $P < 0.05$ ) lower content of DPPH and uric acid compared to the control group.

#### 4. Discussion

Plant extracts may have potential as natural alternatives to antimicrobial growth promoters in animal diets. Tannins have substantial effects on microbial populations and activity (20).

Cross et al. (21) used purified condensed tannin extract from grape seed, and they did not observe a significant effect of the extract (in the amount of 1 g/kg) on live weight (42 days), feed intake, feed:gain ratio, metabolizable energy, or nitrogen digestibility. Tannins can have a negative effect on digestibility and performance, but the effect depends on the structure of the tannins (22).

In this study, inclusion of 1.5% RGP in broiler diets did not have a significant impact on broiler live weight at days 20, 30, and 35 or the overall 35-day feed:gain ratio. Our findings paralleled earlier work by Brenes et al. (12) and Cross et al. (23). In their studies, both Brenes et al. and Cross et al. used grape seed extracts (3.6 g/kg and

**Table 5.** The content of *Escherichia coli* and *Lactobacillus* CFU in grams of digesta of the broilers.

n	C	RGP
	6	6
	Mean ± SE	Mean ± SE
<i>Escherichia coli</i> (CFU × 10 <sup>7</sup> )	6.24 ± 3.513 <sup>a</sup>	2.50 ± 1.040 <sup>a</sup>
<i>Lactobacillus</i> (CFU × 10 <sup>8</sup> )	1.97 ± 0.810 <sup>a</sup>	24.25 ± 9.729 <sup>b</sup>

a, b – means of the same row designated by different letters are significantly different ( $P < 0.05$ ).

SE – standard error.

C – control group without red grape pomace, RGP – group with 15 g/kg red grape pomace.

**Table 6.** Antioxidative potential of liver and blood sera of broilers.

	C	RGP
n	6	6
	Mean ± SE	Mean ± SE
Liver		
MT (µM)	9.1 ± 0.68 <sup>a</sup>	11.6 ± 1.78 <sup>a</sup>
GSH (µM)	17.9 ± 1.19 <sup>a</sup>	16.2 ± 1.90 <sup>a</sup>
Blood sera		
MT (µM)	1.94 ± 0.03 <sup>a</sup>	1.97 ± 0.032 <sup>a</sup>
GSH (µM)	11.21 ± 1.231 <sup>a</sup>	7.70 ± 1.528 <sup>a</sup>
GSSG (µM)	2.35 ± 0.161 <sup>a</sup>	1.92 ± 0.209 <sup>a</sup>
Uric acid (mmol/L)	467.6 ± 45.4 <sup>a</sup>	236.5 ± 33.4 <sup>b</sup>
Free radicals (µg/g)	3.93 ± 0.090 <sup>a</sup>	3.83 ± 0.049 <sup>a</sup>
DPPH (µg/g)*	1.05 ± 0.227 <sup>a</sup>	0.23 ± 0.073 <sup>b</sup>

a, b – means of the same row designated by different letters are significantly different ( $P < 0.05$ ).

SE – standard error.

MT– metallothionein, GSH – reduced glutathione, GSSG – oxidized glutathione.

\*DPPH – diphenylpicrylhydrazyl.

C – control group without red grape pomace, RGP – group with 15 g/kg red grape pomace.

1 g/kg, respectively), and they did not observe a negative impact on broiler performance (weight gain, feed intake, and feed:gain ratio).

In the present study, a significant effect of RGP on protein digestibility was not found. Similarly, Brenes et al. (23), who used GP concentrate (GPC) in broiler diets, did not observe negative effects on performance or crude protein digestibility in broilers fed diets that included GPC at levels up to 60 g/kg up to 42 days of age. Goni et al. (13) also reported no negative effects from dietary GP (30 g/kg) on performance, protein, or amino acid digestibility. However, in our study a positive effect of RGP on digestibility of Thr, Ser, Pro, Gly, and metabolizable energy ( $P < 0.05$ ) was observed. Brenes et al. (12) observed significantly higher ileal protein digestibility in broilers fed 3.6 g/kg grape seed extract at 21 days of age but not at 42 days of age; however, they used both extract and a higher level of extract in comparison with the present study.

The lack of negative effect may be attributed to the low content of polyphenols in the diets. Brenes et al. (23) also did not observe any effect of GP concentrate up to a 30 g/kg level on the digestibility of protein and fat, but

they found significantly lower digestibility of fat when they added 60 g/kg of GP concentrate to the diet. Vitti et al. (22) concluded that it is not possible to predict beneficial or harmful nutritional effects from total tannin concentrations, and in vivo trials are essential to obtain information on intake, CP digestibility, N retention, and live weight gain.

The significantly higher apparent digestibility of Ser and Gly in the RGP group may be related to a significant decrease in uric acid in the blood sera of this group. A significant decrease in uric acid in the excreta after the addition of Gly (Glu) has been observed by Namroud et al. (24). A high level of uric acid indicates an excess of nitrogen compounds that the broiler needs to eliminate (25). The excess of nitrogen can also be caused by an imbalance between energy and nitrogen content in the diet. RGP significantly increased the apparent metabolizable energy, which may also contribute to a decrease in the uric acid level.

RGP had a positive effect on the content of *Lactobacillus* in the lower part of the ileum, but the number of *Escherichia coli* CFUs was not affected by RGP. In contrast, Viveros et al. (15) observed higher populations of *Lactobacillus* in the ileum of birds fed control diets without grape products and birds fed grape seed extract in comparison to birds fed diets with GP concentrate or avoparcin. However, Viveros et al. (15) did not observe an effect of grape products on the *Escherichia coli* population in the ileum. Tannins inhibit microfloral activity and may be selective in their effects based on the concentration of cecal volatile fatty acids, as suggested by Cross et al. (21). Viveros et al. (15) found higher populations of *Lactobacillus* in the cecal digesta in birds fed both GP concentrate and grape seed extract. A possible explanation for the stimulatory effect of polyphenolic compounds on bacterial growth is that some microorganisms are able to use these compounds as nutritional substrates.

The digestibility of polyphenols depends on age, polyphenol type, and dietary polyphenol amount. The polyphenol digestibility range at 42 days of age is from 61 to 69% (12). Brenes et al. (12) observed a significant increase in antioxidant activity both in diets and excreta, depending on the dose of grape seed extract in the diets. The digestibility of hydrolyzable polyphenols is higher than that of condensed tannins (23). These authors reported a positive effect of GPC on sera antioxidant activity after feeding more than 30 g/kg GPC. In contrast, Lafka et al. (3) did not find a correlation between antioxidant activity and total phenol content in winery wastes, but they showed that the antioxidant activity is significantly affected by drying temperatures higher than 60 °C. Moreover, Goni et al. (13) reported a significant effect of 30 g/kg GP on the antioxidant activity in excreta ( $P < 0.05$ ), as determined

by FRAP or ABTS methods, but they did not observe a significantly positive effect of GP on sera antioxidant activity (ABTS method). In the present study, there was no significant effect of RGP on liver or sera antioxidant activity expressed by MT or GSH content in the liver or by MT, GSH, GSSG, or free radical content in the sera. There was significantly lower ( $P < 0.05$ ) DPPH only in the sera of broilers fed RGP. The small effect of RGP on sera antioxidant activity might result from the small amount of extractable polyphenols (13), and this might also explain the positive effect of RGP on *Lactobacillus* in the ileum. The different results can be caused by differences in the qualities of phenolic compounds. Rockenbach et al. (2) and Yi et al. (4) showed that there are significant differences in antioxidant activity among grape varieties. In their study, pomaces of the red wine vinification of

Cabernet Sauvignon and Bordeaux varieties showed the greatest potential as sources of antioxidant compounds and natural colorants, respectively (2).

In conclusion, RGP at a concentration of 15 g/kg in the diet did not have a negative effect on growth. RGP significantly increased the apparent ileal digestibility of several amino acids and the amount of metabolizable energy. The RGP also had a positive effect on the number of *Lactobacillus* in the ileum and on blood antioxidant activity as expressed DPPH. RGP may be beneficial as an alternative to dietary antimicrobial growth promoters.

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