EFFECT OF GRAPE SEED ON ACIDOSIS AND RUMEN GASES IN *IN VITRO* CONDITIONS

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Abstract. Grape seed is a product that is released as waste during the processing of grapes. It is described as ecological damage. Recently, its availability in animal nutrition has been discussed due to the high content of tannins and phenolics contained in it. It is thought that grape seeds can reduce the adverse conditions that occur in the rumen with the high amount of antioxidant properties they contain. This study investigated the effects of grape seed on acidosis and gas production in the rumen under in vitro conditions. The nutritional composition of the present product, *in vitro* gas production (GP), and the differentiation of gases formed in the rumen (NH_3, CH_4, and CO_2) under acidosis conditions were determined. The pH values of the rumen fluid were measured at the 0, 24, 48, and 96th hours of incubation. Grape seed did not have an effect on rumen pH under normal conditions. However, in the rumen, due to its high amount of antioxidant properties during periods of acidosis, it showed a positive effect on the linear improvement of intra-rumen pH. It was concluded that further studies should be conducted, in which the mechanism of action of grape seed on animals with acidosis was studied.
Keywords: *grape seed, acidosis, in vitro gas production, rumen gases*

Introduction

Grape is the fruit of the plant called grapevine *(Vitis, Vitaceae*). Fresh grape production was 77.1 million tons in the world and 3.9 million tons in Turkey based on FAO (2021). In Turkey, in 2019, 1.9 million tons of grapes were produced for table use, 1.5 million tons for drying, and 500 thousand tons for wine (TURKSTAT, 2020). 20% of grapefruit is grape seed (Clifford, 2000). During the processing of grapes, a large amount of grape pulp and seed emerges. In the light of the calculations in TURKSTAT (2020) data, when grape production and export are taken into account, approximately 265 thousand tons of grape seeds are released, which constitutes approximately 14% of grape production. While some of the grape seeds are processed to obtain oil, a large part of them is thrown into nature, causing ecological pollution.

Grape seeds are rich in antioxidant activity. Grape seed oil is rich in linoleic acid, oleic acid and proanthocyanidins, which explains its high antioxidant level (Felhi et al., 2016; Sensoy et al., 2020). The phenolic content of grape seed varies between 71.19-87.03 (mg, GAE/kg) (Akın and Altındişli, 2010). Catechins are the most abundant phenolic compounds in grape seeds, while gallic acid, ferulic and caffeic acid are other antioxidants found in grape seed oil (Lutterodt et al., 2011). Tocopherols oils are the most important sources of antioxidants in the α, β, γ and δ forms found in grape seeds (Kreps and Ark., 2014). Grape seed is used in animal nutrition due to its phenolic compounds. The antimicrobial effect of grape seed is one of the reasons for its use in animal nutrition (Oztürk et al., 2011).

Studies have shown that some plants and their extracts can be used in diseases related to nutrition for ruminant animals (Demirtaş, 2013). Acidosis is the most common disease

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in ruminant animals. Acidosis occurs as subacute or chronic (pH 5.0–5.6), and acute (pH 5.0 and below), depending on the amount, frequency, and Time of consumption of easily digestible carbohydrates. Ultimately, it is a serious nutritional disease that can result in the death of the animal (Nagaraja and Titgemeyer, 2007). Protozoans, which are very sensitive in the rumen microflora, cannot live below pH 5.5 (Krause and Oetzel, 2006). It is a result of the suppression of the growth of bacteria and protozoa that ferment lactic acid in an acidic environment, while stimulating the growth of bacteria that produce acid-resistant lactic acid (Oztürk and Pişkin, 2009).

In this study, the effect of grape seed on acidosis and gases formed in rumen under *in vitro* conditions were investigated.

Material and Method

The plant material of the study consisted of grape seeds taken from Tekirdag Viticulture Research Institute. Grape seeds were ground in a mill with a sieve diameter of 1 mm and used in the analysis. Dry matter (DM) contents of grape seeds were determined by drying in an oven at 105ºC for 4 hours, and ash content was determined by burning in a muffle furnace at 550ºC for 4 hours. Crude protein (CP), Ether extract (EE), and crude cellulose (CC) analyses were made according to the method reported by AOAC (1990). The contents of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL), which make up the cell wall components, were determined by the method reported by Van Soest et al. (1991).

The total flavonoid content of grape seeds was determined according to the method reported by Zhishen et al. (1999). The free radical scavenging efficiency test was carried out using the 1,1-diphenyl-2-picrylhydrazil (DPPH) radical according to the method reported by Blois (1958). ABTS radical scavenging activity was performed according to the method reported by Re et al. (1999).

In Vitro Gas Production Technique

The groups were divided into two groups with grape seed and without grape seed (Control). Then, a mixture of buffer and rumen fluid with standard and acidosis pH levels with the chemicals specified in *Table 1* was added to the groups. In this way, the effect of grape seed under acidosis conditions can be seen. In the study, in which the *in vitro* gas production technique was used, 200 mg of feed samples were weighed into glass syringes as 3 replications for each feed and application. Then, 30 ml of rumen fluid and buffer (10 ml rumen fluid + 20 ml buffer) with normal pH (Normal, 6.80) and low pH (Acidosis, 5.75) was added to glass syringes. The control group contains only rumen fluid and buffer. All syringes were incubated at 39ºC in the incubation cabinet. *In vitro* gas amounts were determined at 3, 6, 12, 24, 48, 72 and 96 hours of incubation (Menke et al., 1979). Grape seed organic matter digestibility (OMD, %), metabolic energy (ME, MJ/kg DM) and net energy lactation (NE_L, MJ/kg DM) values were calculated using the equation reported by Menke and Steingass (1988). The pH value of the rumen fluid was measured with "WTW Inolab pH 730" digital pH meter at 0, 12, 24, 48 and 96 hours of incubation. Ammonia (NH_3), methane (CH_4) and carbon dioxide (CO_2) gases were measured with the MX6 IBRID Multi-Gas Detector at the 48th and 96th hours of the incubation.

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Table 1. *Chemicals used in buffer solution*

Chemicals	Standard (gr/L)	Acidosis (gr/L)
Na_2HPO_4	1,368	0,228
KH_2PO_4	1,488	-
$MgSO_4.7H_2O$	0,144	-
$CaCl_2.2H_2O$	0,01584	-
$MnCl_2.4H_2O$	0,012	-
$CoCl_2.6H_2O$	0,0012	-
$FeCl_2.6H_2O$	0,00096	-
$NaHCO_3$	8,40	0,0525
$(NH_4)HCO_3$	0,96	0,006
Resazurin	1,22	-
1 N NaOH	2,00	-
$Na_2S.7H_2O$	0,336	-

Statistical Analysis

The study was planned according to the 2 x 2 trial design. Statistical analyzes of the data obtained as a result of the analyses were made in the SPSS 22 package program. In the statistical evaluation of the data, a one-way analysis of variance was used to determine the difference between groups, and Duncan's multiple comparison test was used to compare group effects. Pearson for the determination of the relationship between the parameters correlation analysis test was performed (Efe et al., 2000).

Findings and Discussion

Nutrition Value, OMD, ME, NE_L, and Antioxidant Content of Grape Seed

The analysis results regarding the nutrition value, (%DM), OMD, ME, NE_L and antioxidant content of grape seed are given in *Table 2*.

Table 2. *Nutrient content (%DM), OMD, ME, NE_L and antioxidant content of grape seed*

Nutrient Content	
DM	92.22
ASH	2.83
CP	9.18
EE	7.65
CC	34.29
NDF	52.09
ADF	38.68
ADL	34.19
***In Vitro* Parameters**	
OMD (%)	32.70
ME (MJ/kg, DM)	4.90
NE_L (MJ/kg, DM)	2.73
Antioxidant Content	
TPS (GAE/kg)	89.1
DPPH (trolox/g)	265.5
ABTS (trolox/g)	973.5

DM: Dry matter, CP: Crude protein, EE: Ether extract, CC: Crude Cellulose, NDF: Neutral detergent insoluble fiber, ADF: Acid detergent insoluble fiber, ADL: Lignin insoluble in acid solvents, OMD: Organic substance digestibility, ME: Metabolic energy, NE_L: Net energy lactation, TPS: Total amount of phenolic substance (g), DPPH: (µmol), ABTS: (µmol)

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In *Table 3*, there is a difference between the chemical components compared to the studies on grape seed. In this study, DM, ash, CP values were similar to the studies done with grape seed. However, it was found to be lower than other studies in terms of EE and cell wall components. The phenolic value of grape seed was found to be similar to the studies performed (Akın and Altındişli, 2010).

Table 3. *Studies on grape seed*

Chemical Composition (%)	Spanghero et al., 2009	Kilic and Abdiwali, 2016	Choosing, 2017	Altop et al., 2018
DM	-	89.5	90.98-90.21	-
Ash	-	3.8	3.33-3.35	-
CP	12.5	11.1	7.99-8.7	10.13
EE	11.7	13.6	-	12.5
CC	-	51.5	-	47.43
NDF	54.2	64.8	-	64.01
ADF	51.4	56.2	-	57.14
ADL	43.7	52	-	-

DM: Dry matter, CP: Crude protein, EE: Ether extract, CC: Crude Cellulose, NDF: Neutral detergent insoluble fiber, ADF: Acid detergent insoluble fiber, ADL: Lignin insoluble in acid solvents

In Vitro Gas Production Parameters

Analysis results regarding *in vitro* gas production amounts are given in *Table 4* and *Figure 1*.

Table 4. *In vitro gas production amounts of grape seed under normal and acidosis conditions (ml/200mg)*

		Incubation Time (h)						
Condition	**Additive**	**3**	**6**	**12**	**24**	**48**	**72**	**96**
Acidosis	**Control**	0,005^{c}	0,005^{d}	0,755^{d}	1,755^{d}	5,755^{d}	7,505^{d}	8,005^{d}
	Grape seed	2,005^{b}	2,005c	3,005^{c}	5,005^{c}	8,005^{c}	8,505^{c}	10,00^{c}
Normal	**Control**	2,005^{b}	5,255^{b}	15,00^{b}	19,00^{b}	23,00^{b}	26,00^{b}	27,50^{b}
	Grape seed	4,005^{a}	10,005^{a}	22,95^{a}	38,37^{a}	44,37^{a}	48,37^{a}	49,87^{a}
SEM		0,426	1,139	2,724	4,352	4,645	5,005	5,803
				P				
Condition		0,000	0,000	0,000	0,000	0,000	0,000	0,000
Additive		0,000	0,000	0,000	0,000	0,000	0,000	0,189
Condition*Additive		1,000	0,000	0,000	0,000	0,000	0,000	0,330

$^{a-d}$: The difference between groups containing different letters in the same column is statistically significant. SEM: standard error mean

The amount of gas production reached the highest value in the grape seed added group under normal conditions and was found to be statistically significant (P<0.001). Although gas production in acidosis conditions increased slowly after 96 hours of incubation, nutrient degradation and gas production in grape seed were higher than the control group and were found to be statistically significant (P<0.001). It is thought that grape seed procyanidin extract can significantly reduce gas and methane production in the rumen (Zhang, 2020). In a study with grape seed, it was found to reduce methane production by 7-9% (Sinz et al., 2019), and in another study by 28% (Wischer et al., 2013). In a study conducted in the *in vitro* Rusitec system, the effects of grape seed with different doses

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were examined. It was observed that there was an increase in daily production of acetate, butyrate and total volatile fatty acids (TVFA) (Öztürk et al., 2011).

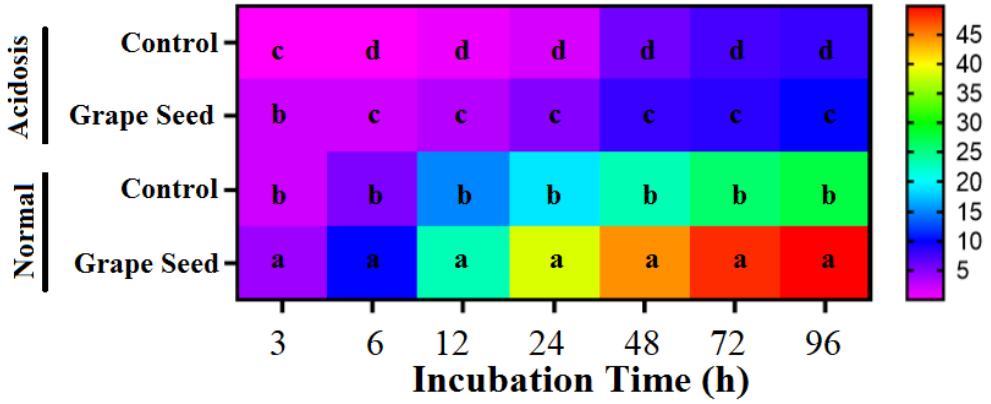

Figure 1. *Grape seed extraction under normal and acidosis conditions; in vitro gas production amounts (ml/200mg)*

The analysis results of the gases released from the rumen fluid are given in *Table 5*.

Table 5. *Analysis results of the gases released from the rumen fluid*

Time (h)	Condition	Additive	NH_3	CH_4	CO_2
48	**Acidosis**	**Control**	3.67^{g}	1.37^{h}	2.85^{h}
		Grape seeds	2.92^{h}	1.86^{f}	3.41^{g}
	Normal	**Control**	51.74^{c}	1.43^{g}	9.09^{d}
		Grape seeds	40.27^{d}	2.29^{e}	24.08^{b}
96	**Acidosis**	**Control**	30.55^{e}	2.88^{d}	5.73^{f}
		Grape seeds	12.42^{f}	3.58^{c}	6.91^{e}
	Normal	**Control**	117.8^{a}	4.22^{b}	13.83^{c}
		Grape seeds	101.52^{b}	6.11^{a}	43.13^{a}
SEM			10,532	0.392	3,327
				P	
Time			0,000	0,000	0,000
Condition			0,000	0,000	0,000
Additive			0,000	0,000	0,000
Time*Condition			0,000	0,000	0,000
Time*Additive			0,000	0,000	0,000
Condition*Additive			0,000	0,000	0,000
Time*Condition*Additive			0,000	0,000	0,000

$^{a-h}$: The difference between groups containing different letters in the same column is statistically significant. NH_3: ammonia (100 ml/mg), CH_4: methane (ml), CO_2: carbon dioxide (ml), SEM: standard error mean

The NH_3 value released from the rumen fluid varied between (2.92-117.80ml), increased over time, and was statistically significant ($P<0.001$). The lowest value was found in the acidosis conditions, while the highest value was found in the control group under normal conditions, the difference between the conditions was statistically significant ($P<0.001$).

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The value of CH_4 released from the rumen fluid varied between 1.37-6.11 ml, increased over time and was statistically significant (P<0.001). In acidosis conditions, the CH_4 value formed with the addition of grape seed increased under normal conditions compared to the control group and was found to be statistically significant (P<0.001). This shows that the grape seed decomposes under acidosis conditions and releases CH_4 into the environment. In a study examining the effects of acidosis in the Rusitec system, it was stated that the CH_4 level decreased with the increase in acidity (Eger et al., 2018).

The amount of CO_2 released from the rumen fluid varied between 2.85-43.13 ml, increased over time and was statistically significant (P<0.001). CO_2 production was limited due to the restriction of the functions of rumen microorganisms in acidosis conditions, CO_2 production increased as a result of some nutrient degradation with the addition of grape seed and was found to be statistically significant compared to the control group (P<0.001). In an *in vitro* study, it was determined that the CO_2 level increased with the increase in acidity (Eger et al., 2018).

The results regarding the pH values in the rumen fluid are given in *Table 6* and *Figure 2*.

Table 6. *pH change in the rumen fluid until the 96th hour of incubation*

Time (h)	Condition	Additive	pH
beginning	**Acidosis**	Control	5.76^{f}
		Grape seeds	5.76^{f}
	Normal	Control	6.81^{a}
		Grape seeds	6.81^{a}
24	**Acidosis**	Control	5.39^{i}
		Grape seeds	5.39^{i}
	Normal	Control	6.75^{c}
		Grape seeds	6.75^{c}
48	**Acidosis**	Control	5.61^{g}
		Grape seeds	5.47^{i}
	Normal	Control	6.69^{e}
		Grape seeds	6.73^{d}
96	**Acidosis**	Control	5.61^{g}
		Grape seeds	5.52^{h}
	Normal	Control	6.77^{b}
		Grape seeds	6.73^{d}
SEM			0.108
			P
Time			0,000
Condition			0,000
Additive			0,000
Time*Condition			0,000
Time*Additive			0,000
Condition*Additive			0,000
Time*Condition*Additive			0,000

$^{a-j}$: The difference between groups containing different letters in the same column is statistically significant. SEM: standard error mean

The initial pH values of the rumen fluid were prepared as 5.76 and 6.81 under acidosis and normal conditions, respectively. pH values decreased to 5.39 in the control and grape seed added groups at the 24th hour of incubation in acidosis condition. Under normal conditions, the pH values decreased to 6.75 in the control and grape seed added groups at

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the 24th hour, and the decrease at the 24th hour compared to the initial values was found to be statistically significant in both acidosis and normal conditions ($p<0.001$). At 48th hour, pH value increased to 5.61 in the control group and 5.47 in the grape seed group under acidosis conditions and decreased in the control and grape seed group under normal conditions. pH changes at 48th hour in all groups were found to be statistically significant ($p<0.001$). At the 96th hour of incubation, acidosis conditions didn't change in the control group, but the increase continued in the grape seed group and the pH became 5.52 and was found to be statistically significant ($p<0.001$). Under normal conditions, the pH value of the grape seed group didn't change compared to the 48th hour, while the pH value of the control group increased and was statistically significant ($p<0.001$). The rapid pH decreases at 24 hours in acidosis groups made it difficult to rise during the 96-hour incubation, but the pH values increased linearly with the addition of grape seed.

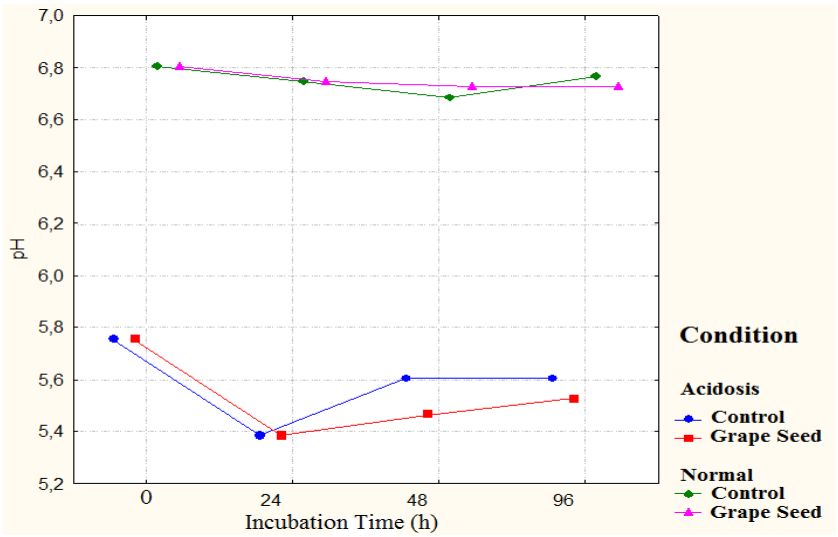

Figure 2. *pH change in the rumen fluid until the 96th hour of incubation*

Under normal conditions, pH decreased to 6.75 in the control and grape seed added group at the 24th hour of incubation, at the 48th hour of the incubation the pH value of the grape seed additive increased compared to the control group and was found to be statistically significant. The increase in the control group exceeded the pH value of the grape seed added group ($p<0.001$). Both conditions didn't reach the initial pH value, it can be said that when the gas production amounts are examined, it can be said that the pH value decreases rapidly with the breakdown of nutrients in the 24th hour. Although grape seed additive didn't affect pH under normal conditions, it increased linearly under acidosis conditions. This shows that grape seed supplement increases rumen pH in acidosis conditions.

In the study where acidosis conditions were created in the Rusitec system, the effect of stinging nettle, chamomile, and chasteberry extracts on acidosis was investigated, these three extracts increased the production of VFA by stimulating the fermentative activities of rumen microorganisms and positively affected the ruminal fermentation efficiency. However, the extracts used in the study didn't show any effect in preventing acidosis (Demirtaş, 2013).

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In light of the data obtained, the findings related to the correlation analysis performed in the control and acidosis groups are given in *Figure 3*.

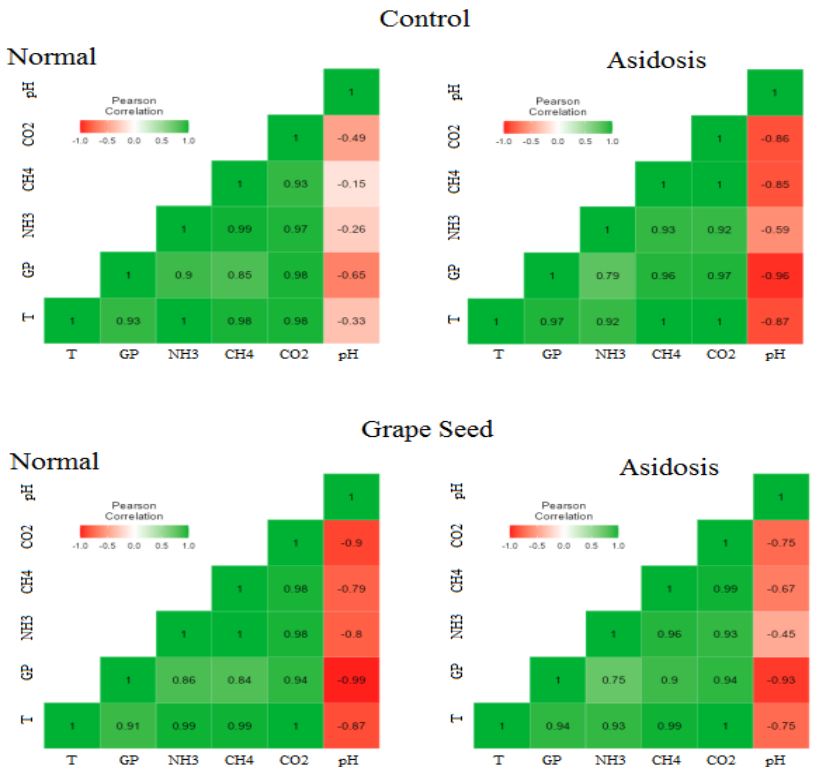

Figure 3. *Findings on correlation analysis*

Depending on the incubation period, the amount of NH_3 increased in the control group under normal conditions, while the amount of CH_4 increased in the control group under acidosis conditions. The amount of CO_2 increased under normal conditions in the other groups except the control group. This increase was similar in the groups with the highest acidosis. Under normal conditions, the amount of NH_3 increased in parallel with the amount of CH_4 with the addition of grape seed, this is due to protein fragmentation.

Conclusion

This study investigated the effects of grape seed on acidosis and rumen gases under in vitro conditions. It is thought that grape seed may be an important feed additive in terms of both animal health and the quality of animal products, with its high antioxidant content. In this study, adverse conditions were created *in vitro* and the effect of grape seed on rumen pH was discussed.

Although the grape seed doesn't affect the rumen pH under normal conditions, it has an effect on the linear increase of the intra-rumen pH due to the high amount of antioxidants under the acidosis conditions.

As a result of this study, it was concluded that the grape seed, which is an ecological waste, can be used as an additive to eliminate the negative conditions in the rumen.

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This study also formed the basis of studies on the acidosis effect of grape seed supplementation at different doses in the rations of ruminant animals with acidosis disease. In order to support this result, it is thought that studies should be multiplied.

Thanks to this study, feeding-related diseases of animals will be added to the studies performed with *in vitro* gas production technique. In this way, problems that will arise before live animal experiments will be seen.

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