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Research Article

Effects of Rumen pH on Degradation Kinetics and Fermentation Indices of Corn Silage Ensiled with Antifungal and Carboxylesterase Producing Inoculants

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ABSTRACT

The present study investigated effects of antifungal and carboxylesterase inoculant on rumen fermentation with different rumen pH. Corn silage was treated without inoculant (CON) and with a mixed *Lactobacillus brevis* 5M2 and *L. buchneri* 6M1 (MIX). Rumen fluid was collected from two cannulated Hanwoo heifers before morning feeding (high rumen pH at 6.70) and 3 h after feeding (low rumen pH at 6.20). Dried corn silage was incubated in the rumen buffer (rumen fluid + anaerobic culture medium at 1:2 ratio) for 48 h at 39°C. Eight replications for each treatment were used along with two blanks. Both in a high and a low rumen pH, MIX silages presented higher (p<0.05) the immediately degradable fraction, the potentially degradable fraction, total degradable fraction, and total volatile fatty acid (VFA) than those of CON silages. Incubated corn silages in a low rumen pH presented lower (p<0.05) total degradable fraction, ammonia-N, total VFA (p=0.061), and other VFA profiles except acetate and propionate, than those in a high rumen pH. The present study concluded that application of antifungal and carboxylesterase inoculant on corn silage could improve degradation kinetics and fermentation indices in the rumen with high and low pH conditions.

(**Key words**: Antifungal, Carboxylesterase, Inoculant, Rumen, Corn silage)

Ⅰ. INTRODUCTION

Whole crop corn is the high energy forage that commonly used to supply the nutritional requirement for beef and dairy cattle (Allen et al., 2003). Whole crop corn is harvested for silage production at mature stage, which contains high concentration of lignocellulose. As generally known, lignocellulose is difficult to degrade in the rumen (Ribeiro et al., 2016). As an increase of lignocellulose concentration, the digestibility of corn silage decrease. According to Ribeiro et al. (2016), application of carboxylesterase was able to breakdown the lignocellulose that could increase the digestibility of dietary fiber in the rumen. Many previous studies were discovered that lactic acid bacteria (LAB) as silage inoculant could produce fibrinolytic enzymes such as carboxylesterase (Jin et al., 2015; Kang et al., 2009; Paradhipta et al., 2019; Paradhipta et al., 2020a)

In our previous study, LAB was isolated from corn silage based on their antifungal activity against *Fusarium graminearum* and carboxylesterase activity. Two potential LAB, *Lactobacillus brevis* 5M2 and *L. buchneri* 6M1, were selected and reported to produce not only antifungal activity, but also carboxylesterase (Paradhipta et al., 2020b). Application of these LAB on farm-scale corn silage was reported to increase fermentation quality and reduce yeast contamination (Paradhipta et al., 2020b). However, the effect of these LAB on rumen fermentation indices of corn silage was not investigated yet. In addition, the antifungal activity from *L. brevis* 5M2 and *L. buchneri* 6M1 might also present the beneficial effects to increase fiber digestibility in the rumen as reported by several previous studies (Russell and Mantovani, 2002; Russell and Rychlik, 2001). In the present study, two different condition of rumen pH were prepared to evaluate the inoculant effect on different animal diets. A high rumen pH

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demonstrated the application of low energy diet, while a low rumen pH demonstrated the application of high energy diet. Both low and high energy diets were used for growing and fattening periods of cattle, respectively. Therefore, the present study was conducted to investigate the effect of dual-purpose inoculant producing antifungal and carboxylesterase activities on rumen degradation kinetics and fermentation indices of corn forage with different rumen pH though *in vitro* study.

Ⅱ. MATERIALS AND METHODS

1. Chemical compositions

Previously, corn forage (Kwangpyeongok hybird) was ensiled for 72 d and treated with two treatments, following: CON, applied destilled water at 1% of fresh forage; and MIX, applied inoculant at 1×10^5 cfu/g of fresh forage. The inoculant contained *L. brevis* 5M2 KACC 92268P and *L. buchneri* 6M1 KACC 92269P (Korean Culture Center of Microorganism, Seoul, Korea) at 1:1 ratio. After ensiling, corn silage was sub-sampled (2 kg) for analyses of chemical composition and rumen fermentation indices.

The sub-sampled corn silage was dried at 65°C for 48 h and ground to pass 1-mm screen using a cutting mill (Shinmyung Electric Co., Ltd, Gimpo, Korea) for the measurement of chemical compositions and rumen fermentation indices. The dry matter (DM) concentration was determined by drying 10 g of sample into the dry oven (OF-22GW, Jeio Tech, Seoul, Korea) at 105°C for 24 h. The crude ash (CA) was determined with a muffle furnace at 550°C for 5 h. According to AOAC (1995), crude protein (CP) and ether extract (EE) were determined by the producers of Kjeldahl using N analyzer (B-324, 412, 435 and 719 S Titrino, BUCHI, Flawil, Switzerland) and Soxhlet, respectively. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by using Ankom 200 fiber analyzer (Ankom Technology, Macedon, NY, USA) following Van Soest (1991). The hemicellulose (HEMI) was determined by calculating the differences between NDF and ADF.

Silage pH and ammonia-N were measured using pH meter (SevenEasy, Mettler Toledo, Greifensee, Switzerland) and the colorimetric method described by Chaney and Marbach (1962), respectively. The silage extraction was centrifuged at 5,645 × *g* for 15 min, and then it was collected the supernatant for lactate and volatile fatty acid (VFA) analyses. The concentrations of lactate and VFA were determined using HPLC (L-2200, Hitachi, Tokyo, Japan) fitted with a UV detector (L-2400; Hitachi, Tokyo, Japan) and a column (Metacarb 87H; Varian, Palo Alto, CA, USA) according to the method described by Muck and Dickerson (1988).

2. *In vitro* rumen incubation

The procedure of animal care was approved by animal ethical committee of Gyeongsang National University, Jinju, Korea (GNU-191011-E0050). The rumen fluid was collected from two non-pregnant cannulated Hanwoo heifers before morning feeding (high rumen pH at 6.70) and 3 h after feeding (low rumen pH at 6.20) to collect different rumen pH condition. The cow diets in the present study consisted of rice straw and commercial concentrate mix at 8:2 ratio. The collected rumen fluid was composited, and then filtered via two layers of cheesecloth. Rumen buffer was made by mixing rumen fluid with anaerobic culture medium at 1:2 ratio described by Adesogan et al. (2005). Dried sample at 0.5 g was put into incubation bottle and 40 mL of rumen buffer was added. Then, the incubation bottle was gassed with CO_2 and closed tightly to reach anaerobic condition. Eight replication used along with two blanks, thus total 18 bottles was incubated. All incubation bottles were placed for 48 h at 39°C in incubator (OF-22GW, Jeio Tech, Seoul, Korea). Gas pressure was monitored in a computer every 30 min using wireless automated system by ANKOM^{RF} (ANKOM Technology, Macedon, NY, USA) to calculate degradation kinetics in the rumen (Adesogan et al., 2005). Rumen degradation kinetics were calculated using nonlinear regression procedure of Statistical Analysis Sofware (SAS, 2002) to fit with the model of McDonald (1981) following:

$$Y = A + B\ (1 - e^{-c(t-L)}) \text{ for } t > L$$

where A is the immediately degradable fraction; B is the potentially degradable fraction; A + B is total degradable fraction; C is the fractional degradation rate; L is the lag phase; and t is time of incubation (h).

After incubation, bottles were opened and transferred to 50 mL conical tube to separate remains sample and supernatant (rumen buffer) through centrifugation at 2568 × *g* for 15 min (Supra 21k, Hanil Electric Corporation, Seoul, Korea, with rotor A50S-6C No.6) according to Paradhipta et al., (2020a). The supernatant was used to analyze rumen fermentation characteristics such as

pH, ammonia-N, and VFA. The protocol to measure pH, ammonia-N, and VFA was described above.

3. Statistical analysis

The experiment was conducted by a 2 (Inoculant; CON vs. MIX) × 2 (Rumen pH; high at 6.70 vs. low at 6.20) factorial design with eight replicates per treatment and all data were analyzed using PROC MIXED of SAS (SAS, 2002) to test the effects of inoculant, rumen pH, and its interaction (inoculant × rumen pH). The model was $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$, where Y_{ijk} = response variable, μ = overall mean, α_i = the effect of inoculant, β_j = the effect of rumen pH, $(\alpha\beta)_{ij}$ = the interaction effect of inoculant and rumen pH, e_{ijk} = error term. In rumen gas volume, data was analyzed as completely randomized design using PROC GLM for each incubation hour (0, 3, 6, 12, 24, 36, 48) and mean separation was performed by Tukey test procedure of SAS (SAS, 2002). All significant differences and tendency were declared at $p \leq 0.05$ and 0.05.

Table 1. Chemical compositions of corn silage (%, DM)

Item	Treatment[1]	
	CON	MIX
Chemical composition, % DM		
Dry matter	26.8 ± 0.06	26.8 ± 0.15
Crude protein	8.98 ± 0.04	8.96 ± 0.11
Ether extract	4.27 ± 0.17	4.23 ± 0.17
Crude ash	4.69 ± 0.06	4.58 ± 0.07
Neutral detergent fiber	49.3 ± 0.92	45.6 ± 0.52
Acid detergent fiber	27.5 ± 0.55	25.0 ± 0.24
Hemicellulose	21.8 ± 0.46	20.7 ± 0.37
Fermentation Characteristics		
pH	3.80 ± 0.02	3.78 ± 0.01
Ammonia-N, % DM	0.06 ± 0.00	0.05 ± 0.00
Lactate, % DM	13.0 ± 0.15	13.0 ± 0.17
Acetate, % DM	2.09 ± 0.02	2.49 ± 0.09
Lactate: Acetate	6.30 ± 0.09	4.34 ± 0.27

[1]CON, corn silage without inoculation; MIX, corn silage inoculated with mixture of *L. brevis* 5M2 and *L. buchneri* 6M1 at ratio 1:1.

Ⅲ. RESULTS

1. Chemical composition and fermentation characteristics of corn silage

In chemical compositions, concentrations of DM, CP, EE, CA, NDF, ADF, and HEMI from CON silage were 26.8, 8.98, 4.27, 4.69, 49.3, 27.5, and 21.8%, respectively (Table 1). The concentrations of DM, CP, EE, CA, NDF, ADF, and HEMI from MIX silage were 26.8, 8.96, 4.23, 4.58, 45.6, 25.0, and 20.7%, respectively. In fermentation characteristics, pH of CON silage and MIX silage were 3.80 and 3.78, respectively. Concentrations of ammonia-N, lactate, and acetate from CON silage were 0.06, 13.0, and 2.09%, respectively. On the other side, concentrations of ammonia-N, lactate, and acetate from CON silage were 0.05, 13.0, and 2.49%, respectively. The lactate to acetate ratio of CON silage and MIX silage were 6.30 and 4.34, respectively.

2. Rumen degradation kinetics

Application of MIX silages both in a high and a low rumen pH presented higher the immediately degradable fraction (p<0.001; 0.58 vs. 0.28 mL/g), the potentially degradable fraction (p=0.003; 4.39 vs. 4.06 mL/g), and the total degradable fraction (p<0.001; 4.96 vs. 4.34 mL/g) than those of CON silages (Table 2). The fraction degradation rate and the lag phase were not affected by inoculant application. On the other side, application of corn silages in a low rumen pH presented higher the immediately degradable fraction (p<0.001; 0.50 vs. 0.36 mL/g), the fraction fermentation rate (p=0.002; 0.13 vs. 0.11%/h), and the lag phase (p<0.001; 3.50 vs. 1.38 h), with lower the potentially degradable fraction (p<0.001; 3.71 vs. 4.74 mL/g) and the total degradable fraction (p<0.001; 4.21 vs. 5.09 mL/g) than those in a high rumen pH. The interaction effect (p<0.001) between inoculant and rumen pH was found only in the immediately degradable fraction.

3. Rumen fermentation indices

Incubated CON silage and MIX silage in a high rumen pH presented higher (p<0.05) gas volume than CON silage and MIX silage in a low rumen for each incubation hour (Fig. 1). In addition, MIX silage presented higher (p<0.05) gas volume than CON silage in a low rumen pH during 24 h of incubation.

Application of MIX silages both in a high and a low rumen pH presented lower pH (p=0.002; 6.52 vs. 6.58) and higher total gas volume (p=0.049; 175.3 vs. 166.2 mL/g) and total VFA

Table 2. Effects of inoculant application on rumen degradation kinetics of corn silage incubated for 48 h in rumen buffer with different pH

Item[1]	High[2]		Low		SEM	Contrast[3]		
	CON	MIX	CON	MIX		INO	RPH	INO*RPH
A, mL/g DM	0.28	0.43	0.28	0.72	0.044	<.001	<.001	<.001
B, mL/g DM	4.61	4.86	3.50	3.91	0.116	0.003	<.001	0.301
A+B, mL/g DM	4.89	5.29	3.78	4.63	0.084	<.001	<.001	0.341
C, %/h	0.10	0.11	0.12	0.14	0.010	0.093	0.002	0.309
L, h	1.34	1.41	3.48	3.51	0.226	0.695	<.001	0.892

[1]A, the immediately degradable fraction; B, the potentially degradable fraction; A+B, the total degradable fraction; C, the fraction degradation rate; L, the lag phase.
[2]High, rumen pH at 6.70; Low, rumen pH at 6.20; CON, corn silage without inoculation; MIX, corn silage inoculated with mixture of *L. brevis* 5M2 and *L. buchneri* 6M1 at ratio 1:1.
[3]INO, inoculant application effect; RPH, rumen pH effect; INO*RPH, interaction effect between inoculant application and rumen pH.

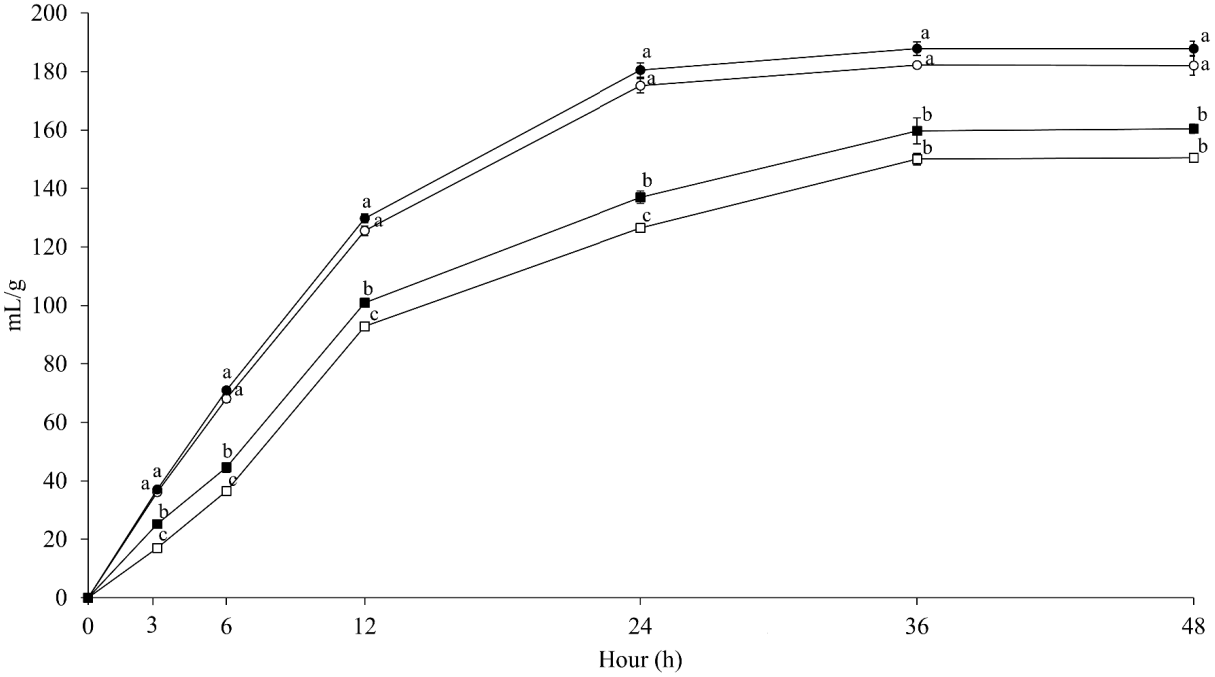

Fig. 1. Effects of inoculant and rumen pH on rumen gas volume of corn silage incubated in rumen buffer for 48 h. Corn silage without inoculant in a high rumen pH (○); Corn silage with inoculant in a high rumen pH (●); Corn silage without inoculant in a low rumen pH (□); Corn silage with inoculant in a low rumen pH (■). [a~c]Means in the same hour with different superscripts differ significantly ($p \leq 0.05$). The bar indicates standard error.

concentration (p=0.027; 161.2 vs. 156.6 m*M*/dL) than those of CON silages (Table 3). Application of corn silages in a low rumen pH presented higher pH (p<0.001; 6.59 vs. 6.51) and concentrations of acetate (p=0.029; 65.3 vs. 64.2% of molar) and propionate (p<0.001; 23.15 vs. 19.85% of molar), but lower total gas volume (p<0.001; vs. 185.6 vs. 155.9 mL/g) and concentrations of ammonia-N (p<0.001; 25.2 vs. 31.8 mg/dL), iso-butyrate (p<0.001; ND vs. 0.65% of molar), butyrate (p<0.001; 9.49 vs. 11.90% of molar), iso-valerate (p<0.001; 2.09 vs. 3.43% of molar), and acetate to propionate ratio (p<0.001; 2.83 vs. 3.24) than those in a high rumen pH. Also, corn silages incubated in a low rumen pH tended to have lower total VFA than in a high rumen pH (p=0.062; 157.1 vs 160.8 m*M*/dL).

Table 3. Effects of inoculant application on rumen fermentation indices of corn silage incubated for 48 h in rumen buffer with different pH

Item	High[1]		Low		SEM	Contrast[2]		
	CON	MIX	CON	MIX		INO	RPH	INO*RPH
Total gas volume, mL/g	181.8	189.3	150.5	161.2	7.284	0.049	<.001	0.709
pH	6.54	6.47	6.61	6.57	0.027	0.002	<.001	0.227
Ammonia-N, mg/dL	32.1	31.5	26.4	24.0	0.906	0.052	<.001	0.194
Total VFA, m*M*/dL	157.5	164.0	155.7	158.4	2.964	0.027	0.062	0.295
Acetate, % molar	64.5	63.9	64.8	65.8	0.800	0.480	0.029	0.063
Propionate	19.8	19.9	23.6	22.7	0.953	0.417	<.001	0.339
Iso-butyrate	0.66	0.64	ND[3]	ND	0.047	0.702	<.001	0.680
Butyrate	11.7	12.1	9.57	9.41	0.317	0.612	<.001	0.131
Iso-valerate	3.37	3.49	2.09	2.08	0.137	0.408	<.001	0.390
Acetate: propionate	3.26	3.21	2.75	2.90	0.150	0.525	<.001	0.244

[1]High, rumen pH at 6.70; Low, rumen pH at 6.20; CON, corn silage without inoculation; MIX, corn silage inoculated with mixture of *L. brevis* 5M2 and *L. buchneri* 6M1 at ratio 1:1.
[2]INO, inoculant application effect; RPH, rumen pH effect; INO*RPH, interaction effect between inoculant application and rumen pH.
[3]ND, not detected.

Ⅳ. DISCUSSION

The chemical compositions of CON silage and MIX silage were in the normal range of corn silage according to previous study (Lee et al., 2019). The fermentation characteristics of corn silages both CON silage and MIX silage were also in the normal range based on Kung et al. (2018). The acetate concentration seemed higher in MIX silage than in CON silage. The result of acetate in the present study supported with McDonald et al. (1991) that *L. brevis* and *L. buchneri* were classified as heterofermentative LAB, which could increase acetate concentration in the silage. In rumen, application of inoculant on corn silage increased the potentially degradable fraction, which could be caused by the carboxylesterase activity from *L. brevis* 5M2 and *L. buchneri* 6M1. In addition, the immediately degradable fraction of corn silage also increased with inoculant application in the present study. This result indicated that the presence of carboxylesterase during ensiling could provide more soluble carbohydrate due to the degradation of lignocellulose. Generally, the immediately degradable fraction consists of soluble fraction (Hobson and Stewart, 1997). Besides that, the carboxylesterase activity of inoculants could be shown from rumen gas volume, where incubated MIX silage both with a high and a low rumen pH had higher gas volume in all of incubation hours. According to Ribeiro et al. (2016), carboxylesterase is not produced by rumen microbes, thus the degradation of lignocellulose was limited for ruminants. The presence of carboxylesterase, such as ferulic acid esterase and acetyl esterase, can breakdown the lignocellulose and increase digestibility of dietary fiber in the rumen (Ribeiro et al., 2016). Paradhipta et al., (2020a) also reported the similar results to present study, where application of LAB producing ferulic acid on rye silage increased the immediately degradable fraction and the potentially degradable fraction in the rumen. Not only Paradhipta et al., (2020a), but also the other studies also reported an increasing digestibility of silage in the rumen due to application of carboxylesterase inoculant at ensiling (Jin et al., 2015; Kang et al., 2009; Paradhipta et al., 2019). On the other side, the improvement of rumen degradation kinetics in MIX silage might be also influenced by antifungal activity from *L. brevis* 5M2 and *L. buchneri* 6M1. Several previous studies discovered that antifungal substance produced by LAB inhibited the pathogenic microorganism in the rumen, thus the presence of LAB producing antifungal could improve the fiber digestion. Moreover, the antifungal substance produced by LAB could present a role as antibiotics to increase animal performance (Ruseell and Mantovani, 2002; Russell and Rychlik, 2001).

On the other side, application of a low rumen pH resulted in decreases of the potentially degradable fraction and the total degradable fraction with the increase of the lag phase. These

results of present study supported with the results of Wales et al. (2004). The activity of fibrinolytic bacteria in the rumen was inhibited by low pH condition. Moreover, lower pH can be a cause of acidosis for ruminant (Hobson and Stewart, 1997; Wales et al., 2004). The decrease of fibrinolytic activity by rumen microbes would decrease the feed digestion such as the result of the present study. In addition, the amylolytic and lactic acid bacteria were dominant in a low rumen pH that degraded rapidly the soluble fraction into organic compound, such as propionate or lactate (Hobson and Stewart, 1997). This might be a cause for increases of the immediately degradable fraction and the fractional degradation rate in a low rumen pH. Interestingly, inoculation of mixed *L. brevis* 5M2 and *L. buchneri* 6M1 on corn silage still presented the increase of total digestible fraction although applied with a low rumen pH.

In the rumen fermentation indices, the improvement of VFA concentration in MIX silages supported with the results of total gas volume and total degradable fraction. This indicated that carboxylesterase produced by *L. brevis* 5M2 and *L. buchneri* 6M1 could increase digestibility and rumen fermentation indices. This result of present study was in agreement with Paradhipta et al., (2020a) that reported the improvement of total VFA concentration due the application of LAB producing ferulic acid esterase at ensiling of rye silage. MIX silages presented lower rumen pH due to higher concentration of VFA than CON silages, which supported with Hobson and Stewart (1997).

Similar with the result of degradation kinetics, application of a low rumen pH decreased rumen fermentation indices such as total gas volume and concentrations of ammonia-N, total VFA, and all individual VFA profiles, except acetate and propionate. Moreover in each hour of incubation, silage incubated in a low rumen pH had lower gas volume than incubated in a high rumen pH. It indicated that rumen condition with low pH decreased the digestibility of corn silage. As explained before, activity of fibrinolytic bacteria to degrade corn silage fraction was inhibited with low rumen pH (Hobson and Stewart, 1997; Wales et al., 2004). Increased propionate in a low rumen pH was caused because the amylolytic bacteria were dominated rumen ecosystem with low pH (Hobson and Stewart, 1997). A decrease of butyrate concentration might be a reason for an increase of acetate concentration in a low rumen pH (Hobson and Stewart, 1997; Sutton et al., 2003).

The present study concluded that application of dual-purpose inoculant containing *L. brevis* 5M2 and *L. buchneri* 6M1 on corn silage could improve degradation kinetics and fermentation indices in the rumen both with high and low pH conditions using *in vitro* technique. Generally, a low rumen pH decreased degradation kinetics and fermentation indices because the activity of fibrinolytic was inhibited.

Ⅴ. ACKNOWLEDGMENT

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