

Effect of nutmeg essential oil (*Myristica fragrans* Houtt.) on methane production, rumen fermentation, and nutrient digestibility in vitro

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Abstract

The study evaluated the effect addition of nutmeg (*Myristica fragrans* Houtt.) essential oil as a feed additive on methane production, rumen fermentation parameters, rumen enzyme activity, and nutrient digestibility in vitro. This study was divided into three treatments based on the level addition of nutmeg essential oil, including 0 µL/L (T0), 100 µL/L (T2), and 200 µL/L. The composition of feed substrate was king grass as forage and concentrate with a ratio of 60:40. Feed fermentation was carried out using the Menke and Steingass gas production and two-stages Tilley and Terry in-vitro digestibility technique. The data obtained from the study were analyzed using one-way ANOVA and If there were differences between means continued by DMRT. Results showed that 200 µL/L nutmeg essential oil addition significantly decreased ($P < 0.05$) ammonia (NH_3) levels, VFA total, acetate, propionate, butyrate, and microbial protein ($P < 0.05$). Methane production and activity of rumen protease enzyme significantly decreased ($P < 0.05$) with levels at 100 and 200 µL/L. The addition at 200 µL/L of nutmeg essential oil significantly decreased ($P < 0.05$) on protein digestibility (IVCPD) in 48h, while IVCPD in 96 h was significantly increased ($P < 0.05$). on the other hand, the addition of nutmeg essential oil did not affect on the activity of the amylase, carboxymethyl cellulase, and β -glucosidase enzymes and in vitro digestibility of dry matter (IVDMD), crude fiber (IVCFD), and organic matter (IVOMD). The conclusion obtained from this study was the optimum level at 200 µL/L of nutmeg essential oil could reduce methane production and increased crude protein digestibility in 96 h without any negative effect on rumen fermentation and nutrient digestibility.

Introduction

Ruminant farming is one of the animal protein sources with high nutritional values for human consumption, such as meat and milk. However, ruminant farming cause a negative impact on environmental problems, namely global warming due to the production of methane gas (CH_4)¹. CH_4 is the second-most significant greenhouse gas (GHG) after carbon dioxide. The methane gas contributes to the atmosphere of greenhouse gas (GHG), which have potential to contribute in global warming (16–25% of total GHG)². Agriculture is the main contributor to global CH_4 emission caused by human activities. Approximately 80% of agricultural CH_4 is produced by livestock systems, with nearly 90% coming from enteric fermentation by ruminants such as cattle and sheep and the remaining 10% coming from animal manure³. On the other hand, methane production from enteric fermentation also negatively impacts on the animals. The methane production can reflect the loss of feed energy about 3–12% of digested energy. As a result, the productivity of animals was decreased⁴. Therefore, innovative technology is required to reducing methane emissions from ruminants would reduce national greenhouse gas emissions and increase the available energy for livestock productivity.

Manipulation of the rumen fermentation process such as giving feed additives which will stimulate growth and rumen microbial activity to improve digestibility and efficiency of feed nutrients⁵. Synthetic feed additives in ruminant production, such as antibiotics, ionophores, methane inhibitors, and defunct agents, have been commonly used to increase meat and milk production, improve consumption, and improve feed efficiency and disease prevention⁶. The use of antibiotics as feed additives has become the focus of attention and prohibition in various countries, especially Indonesia, due to health aspects. So alternative feed additives are needed to optimize livestock production, such as probiotics and phytobiotics⁷. Secondary metabolite extracts from plants, such as saponins, tannins, and essential oils have been evaluated for enhancing microbial metabolism in the rumen, increasing efficiency fermentation, and inhibition methanogenesis production⁸.

Nutmeg (*Myristica fragrans* Houtt.) is a leading commodity in the tropics, including Indonesia as the world's largest nutmeg producer. Nutmeg is widely used in scale industry to produce essential oils through steam distillation process of nutmeg seeds. Steam distillation of nutmeg seeds contains several bioactive compounds including sabinene (21.38%), 4-terpineol (13.92%), and myristicin (13.57%)⁹. In the current study, decreased number of protozoa cells that correlated with reduced of methane production were reported with using essential oil such as myristicin¹⁰ and terpineol¹¹ as an active compound. Although, essential oils have shown potential in inhibiting the methanogenesis in the rumen, negative effects on fiber digestion and fermentation have also been reported, the quantity depends on the type and dose of essential oil and the composition of diet¹². Therefore, this study was conducted to evaluate nutmeg essential oil as feed additive to reducing methane gas by modifying fermentation in the rumen and correlated with increased productivity and feed efficiency.

Results

Effect nutmeg essential oil on fermentation parameters. Based on the data in Table 1, the use of nutmeg essential oil at 100 µL/mL significantly decreased ($P < 0.05$) the concentration of total VFA. The use up to 200 µL/mL significantly decreased ($P < 0.05$) the concentration of NH_3 , acetate, propionate, butyrate, and microbial protein. Nutmeg essential oil was no effect ($P > 0.05$) on pH, ratio of acetate and butyrate, and protozoa cells.

Table 1
Effect of nutmeg essential oil on rumen fermentation parameters

Fermentation parameters	Level of nutmeg essential oil (μL/L)		
	0	100 μL	200 μL
pH	6.87 ± 0.01	6.84 ± 0.02	6.86 ± 0.02
NH ₃ (mg/100mL)	23.34 ± 0.99 ^a	22.42 ± 0.58 ^{ab}	20.93 ± 0.19 ^b
VFA (mM)			
Acetate (mM)	82.34 ± 1.05 ^a	80.50 ± 2.01 ^a	73.77 ± 0.93 ^b
Propionate (mM)	18.86 ± 1.27 ^a	16.85 ± 0.72 ^{ab}	15.56 ± 0.70 ^b
Butyrate (mM)	7.70 ± 0.26 ^a	7.15 ± 0.53 ^a	5.84 ± 0.30 ^b
Acetate: Propionate	4.38 ± 0.25	4.78 ± 0.17	4.75 ± 0.27
Total VFA (mM)	113.47 ± 3.47 ^a	96.71 ± 2.26 ^b	95.19 ± 3.80 ^b
Microbial protein (mg/mL)	1.10 ± 0.04 ^a	1.07 ± 0.05 ^a	0.98 ± 0.01 ^b
Protozoa (selx10 ³)	2.72 ± 0.17	2.66 ± 0.13	2.63 ± 0.26
^{a, b} Different superscripts in the same line show a significant effect (P < 0.05)			

Effect nutmeg essential oil on methane production. The methane production is given in Table 2. The methane production significantly decreased (P < 0.05) with using of nutmeg essential oil start from 100 μL/L. Methane reduction was significantly different at level 100 μL/L with the control, but no significantly different with 200 μL/L.

Table 2
Methane production with the using of nutmeg essential oil

Parameter	Level of nutmeg essential oil (μL/L)		
	0	100 μL	200 μL
Methane (mL/300mgDM)	14.83 ± 0.48 ^a	12.15 ± 0.78 ^b	11.65 ± 1.14 ^b
Methane/DMD (mL/g)	92.58 ± 2.99 ^a	77.12 ± 5.47 ^b	75.60 ± 7.67 ^b
Methane/OMD (mL/g)	93.58 ± 2.15 ^a	78.25 ± 6.66 ^b	75.71 ± 8.28 ^b
^{a, b} Different superscripts in the same line show a significant effect (P < 0.05)			

Effect nutmeg essential oil on rumen enzymes activity. The activity of the protease enzyme was significantly decreased (P < 0.05) with the using of nutmeg oil essential oil start from 100 μL/mL, while the using at level 200 μL/L has no significantly different with 100 μL/L. Nutmeg essential oil was no effect (P > 0.05) in the activity of the amylase, carboxymethyl cellulase, and β-glucosidase enzymes (Table 3).

Table 3
Rumen enzymes activity with the using of nutmeg oil essential oil

Enzyme Activity	Level of nutmeg essential oil (μL/L)		
	0	100 μL	200 μL
Amylase (U/g) ^{ns}	27.86 ± 2.69	27.83 ± 2.48	26.69 ± 2.91
Carboxymethyl Cellulase (U/g) ^{ns}	1.71 ± 0.09	1.70 ± 0.15	1.51 ± 0.12
β-Glucosidase (U/g) ^{ns}	55.13 ± 3.44	54.41 ± 1.83	51.40 ± 1.99
Protease (U/g)	63.03 ± 1.47 ^a	56.35 ± 2.34 ^b	52.12 ± 1.57 ^b
^{a, b} Different superscripts in the same line show a significant effect (P < 0.05)			

Effect Nutmeg essential oil on nutrient digestibility. As shown in Table 4, the effect of using nutmeg essential oil at level 200 μL/L significantly decreased (P < 0.05) on protein digestibility (IVCPD) in 48h, while IVCPD in 96 h was significantly increased (P < 0.05). Nutmeg essential oil has no effect (P > 0.05) on in vitro digestibility of crude fiber (IVCFD), organic matter (IVOMD), and dry matter (IVDMD) in 48 and 96 h incubation.

Table 4
In vitro digestibility of feed nutrients with the using of nutmeg essential oil

Nutrient digestibility	Level of nutmeg essential oil (μL/L)		
	0	100	200
Crude Protein			
48 h	46.68 ± 0.32 ^a	45.39 ± 0.57 ^{ab}	44.24 ± 0.7 ^b
96 h	60.95 ± 0.57 ^b	62.09 ± 0.47 ^{ab}	63.54 ± 1.06 ^a
Crude Fiber			
48 h	38.94 ± 0.29	38.59 ± 1.05	37.40 ± 0.89
96 h	47.04 ± 1.38	48.35 ± 0.89	49.30 ± 0.51
Organic Matter			
48 h	53.00 ± 1.53	52.25 ± 1.90	50.70 ± 0.89
96 h	50.55 ± 0.54	51.10 ± 0.26	53.20 ± 1.73
Dry Matter			
48 h	44.33 ± 2.04	43.20 ± 2.21	42.67 ± 2.23
96 h	52.53 ± 1.32	53.13 ± 1.32	55.13 ± 0.57
^{a, b} Different superscripts in the same line show a significant effect (P < 0.05)			

Discussion

Ammonia is produced in the rumen result of feed protein degradation by rumen microbes as a protein source for synthesizing amino acids and microbial cells¹³. Rumen ammonia (NH₃) content reflect the degradation activity of feed protein and endogenous protein by rumen microbes through the N balance mechanism of the livestock body⁸. The higher ammonia levels are the results of an increase in amino acid deamination is dominated by HAP (Hyper Ammonia Producer) bacteria. There is excessive formation of ammonia in the rumen, which is not utilized by livestock and will be excreted through the urine in the form of urea⁴. In the current study, using nutmeg essential oil at 200 μL/L decreased ammonia concentration by 10.33% of the control. In similar regard that rosemary essential oil at 250 μL decreased ammonia concentration in the rumen by 44.88% from the control¹⁴. The decrease in ammonia concentration was influenced by active compounds in essential oils that were reactive to hyper ammonia producer (HAP) bacteria, namely phenolic compounds⁴. Essential oils have an anti-microbial activity which reduce the rumen microbial population of the HAP which causes excess ammonia production in the rumen and disrupts the *Prevotella* bacteria which have a proteolytic activity¹⁵. Rumen ammonia concentration ranges from 0-130 mg/100 mL, but the optimal concentration for microbial protein synthesis ranges from 5.6–10.0 mg/100 mL when energy available in the rumen¹⁶. Ammonia concentration in this study were normal for microbial protein synthesis.

Nutmeg essential oil at 200 μL/mL decreased total VFA production by 15.75% of the control. The decrease in total VFA in the rumen was influenced by active compounds in the nutmeg essential oil and the doses used in the treatment. In the same line, using tea tree essential oil starting from 125 μL/mL with active compounds such as terpinen-4-ol, γ-terpinene, α-pinene, cymene, α-terpineol, and terpinolene decreased total VFA¹³. The chemical composition of essential oils, dosage use, and feed ration composition can affect total VFA production in the rumen. The composition of the active compounds of essential oils in the plant of origin varies¹⁵. Essential oils from various plants at high doses largely reduced total VFA production and reflected a decrease in feed degradation, especially on microbial that ferment carbohydrate in the rumen to VFA production that used by animal host as the main energy source¹⁷. The antibacterial effect of essential oils such as terpenoid and phenylpropanoid compounds are hydrophobic and cyclic hydrocarbons, which interact with cell membranes and the lipid layer of bacteria, resulting in cell membrane leakage and cell death¹⁸. The normal concentration of VFA is 70 to 150 mM which needs for microbial protein synthesis⁴. This study obtained VFA concentrations ranging from 95.19 to 113.47 mM that normal range for microbial protein synthesis.

In the current study, the concentration of microbial protein was decreased by using of nutmeg essential oil at 200 μL/mL by 10.91% of the control. The decrease of microbial protein concentration in this study correlated with reduced NH₃ and total VFA with using nutmeg essential oil at 200 μL/mL (Table 1). Microbial protein is the result of fermented feed in the rumen as a source of protein for ruminants. The substrate used in the formation of microbial proteins is ammonia as a N source and VFA as an energy source. Ammonia is used by rumen microbes in the microbial protein synthesis¹⁹. The formation of microbial protein depends on the availability of feed nitrogen. Substrate used in the formation of microbial protein is ammonia derived from the degradation of feed protein, feed NPN, or ammonium salts⁴. Moreover, microbial protein synthesis also depends on supply of adequate amounts and type of CHO as an energy source for the synthesis of peptide bonds. Readily fermentable CHO, such as starch or sugars, are more effective than other CHO sources, such as cellulose, in promoting microbial growth²⁰

The results of this study showed that nutmeg essential oil decreased methane production in rumen fermentation at 100 and 200 μL/mL. In agreement with this finding, the utilization of oregano essential oil with an active compound, namely eugenol starts from 13 μL/L reduced methane production, where the decreased in methane was linear with increased levels of addition²¹. Others finding, reported that fennel essential oil at doses of 200 and 400 μL/L resulted in

a high reduction in methane production of 27.03% and 47.75%²². Changes in methane production can be linked to decreased VFA production, especially the proportion of acetic acid in this study (Table 1). Acetic acid formation produces hydrogen gas (H₂) that is used as a substrate in methanogenesis reactions. The decreased of acetic acid formation will reduce substrate for methane production²³. The proportion of propionic acid also decreased significantly at each level usage of nutmeg essential oil (Table 1). Feed additives is able to change the patterns of hydrogen use towards to propionate production which aims to increase the energy efficiency of host livestock and reduce methane gas production²⁴. The proportion of propionic of this study is different with the literature.

Based of this study, the protease enzyme activity significantly decreased with the addition of nutmeg essential oil at 100 and 200 µL/mL by 10.59% and 17.30% of the control. The previous study reported that the addition of galangal essential oil starts from 30 µL/300 mg (DM feed) decreased protease enzyme activity. This study showed that the addition of nutmeg seed oil decreases the activity of the protease enzyme which can reduce the digestibility of crude protein and ammonia concentration in the rumen. The decrease of protease enzyme activity was influenced by active compounds in nutmeg essential oils such as phenols. The mechanism is hydroxyl group of phenolic compounds can bind to enzyme protein. Phenol have sensitivity to the Gram-negative bacteria such as *Prevotella* genus, R. *Amylophilus*, an amylolytic and proteolytic bacteria²⁵. The inhibition of bacteria protease colonization in the rumen by essential oils, which was indicated by a decrease in protease activity²⁶. This study reported that amylase, CMC-ase, and β-glucosidase enzyme activity not influenced by the addition of nutmeg essential oil. In agreement with this finding, the addition of blend essential oil (clove bud oleoresin and lemongrass oil) at 0%, 0.75%, and 1.5% no significant effect on amylase, CMC-ase, and β-glucosidase enzyme activity²⁷. Enzymes catalyze the hydrolysis of proteins, fats, and carbohydrates into simple form. The measured of digestive enzyme activity can describe the ability of feed nutrient utilization²⁸. Nutmeg essential oil contains terpenes, which inhibit the breakdown of cellulose. Terpenes are known to inhibit the growth of rumen microorganisms, which are important for the breakdown of cellulose²⁹. The utilization of nutmeg essential oil up to 200 µL/mL in this study is safe and does not interfere with enzyme activity in the rumen.

Nutmeg essential oil at 200 µL/mL decreased in vitro crude protein digestibility (IVCPD) in the rumen (48 h) by 4.27% from the control. In the same line, previous study reported that the decreased of IVCPD in the rumen by the addition of cinnamon³⁰ and lemongrass³¹ essential oil. The decrease of protein degradation in the rumen related with essential oils have an action mechanism selective antimicrobial against rumen microorganisms, especially proteolytic bacteria which result in decreased activity of rumen protease enzymes³². The addition of nutmeg EO at a level of 200µL/L reduced the activity of protease enzymes in the rumen (Table 3). The action mechanism of essential oils inhibits bacterial hyperammonemia by phenolic compounds can bind to the amino groups of proteins, thereby reducing the deamination of proteins in the rumen²⁶. This study reported that ammonia concentration was reduced that correlated with protease enzyme activity (Table 1). The decreased of IVCPD in this study was also due to a reduced in the abundance of proteolytic bacteria in the rumen. The protease enzyme was secreted by proteolytic bacteria to hydrolyze feed protein that enters the rumen into amino acids which are then deaminated into ammonia³³. The use of nutmeg EO as a source of phenol is also likely to inhibit the growth of proteolytic bacteria thereby reducing protease activity in rumen fluid. The utilization of BEO essential oil blend consisting of thymol, eugenol, vanillin and limonene of 1000 mg/Kg of feed inhibited the growth and activity of several proteolytic bacteria (*Clostridium sticklandii*, *Prevotella* and *Peptostreptococcus anaerobius*) hyperammonia producing bacteria. (HAP)³⁴. The *Prevotella ruminicola* strain had the highest protease activity in the rumen. The bioactive component of nutmeg EO has a toxic effect on proteolytic bacteria. The bioactive components of nutmeg such as phenols and monoterpenes, synergize with each other to provide an antibacterial effect against the abundance of proteolytic bacteria in the rumen³⁵. The others study reported that the addition of eucalyptus and rosemary EO at a dose of both 60 µL with the main components cineol and phenolic compounds significantly reduced total *Prevotella* compared to controls.

Protein is degraded by rumen microbes into peptides and amino acids as intermediate products, some amino acids were further metabolized by rumen microbes into ammonia or use for synthesis microbial protein. At the same time, undigested protein in the rumen will enter the abomasum and intestinal tract to digest by the protease enzymes of the digestive tract, then absorbed and utilized for metabolism. The decrease of crude protein digestibility in the rumen is an advantage that increase the efficiency of feed protein absorption in the intestinal tract of animal host which increase livestock productivity²². The addition of nutmeg EO at 200µL/L significantly increased on the crude protein digestibility at 96 h by 4.24% from the control. This decrease can be attributed to the phenol compound in the nutmeg essential oil. Essential oils have the functional group OH (phenol) with reactive properties to macromolecules such as proteins through the ester bond. The ester bond occurs because there is a hydroxyl group (COOH) on the amino acid to form bonds with the hydroxyl group (OH) of phenol³⁶. Bioactive compounds from essential oils contain phenol functional groups capable of forming bonds with protein to escape rumen degradation, which can be optimally degraded in the post-rumen³⁶

Materials and Methods

Substrate and research design. The substrate was a complete feed consisting of king grass (*Pennisetum purpureum*) as forage and concentrate that mixture of several ingredients (soybean hulls, peanut hulls, rice hulls, wheat bran, rice bran, palm meal, copra meal, corn, cassava peels, cassava flour, cassava cake, corn gluten feed, salt, minerals, and molasses) with a ratio of 60:40. Nutmeg essential oils was purchased from Nutmeg Essential Oil Industry from Ungaran, Semarang, Central Java, Indonesia. Nutmeg essential oil was diluted with acetone to get 0 µL/L (T1), 100µL/L (T2), and 200µL/L (T3) concentrations. The nutmeg essential oil solution was added based on the buffered inoculums in each tube. Acetone was added to the treatment control (without essential oil).

Proximate analysis. The forage was cut into pieces with a size of 2 to 3 cm then dried in an oven 55 °C for three days while concentrate was dried for one night to determine dry matter (DM). The feed ingredients were ground to pass a 1 mm screen. The ground samples were analyzed to determine the value of organic matter (OM), crude protein (CP), and crude fiber (CF)³⁷. The chemical composition of the feed materials used in the present study is shown in Table 5.

Table 5
The chemical composition of the feed materials

Feed material	Chemical composition			
	DM (%)	OM (%)	CP (%)	CF (%)
King grass	91.52	87.79	7.15	30.74
Concentrate	90.63	85.20	11.58	17.80

Preparation of rumen fluid and buffered inoculums. The rumen fluids and buffered inoculums were prepared for gas production, fermentation parameters, and nutrient degradability analysis^{38,39}. The rumen-fistulated female Bali cattle with an average liveweight of 452 ± 18 kg was the donors of rumen fluid. The cattle received of diet were composed of king grass and wheat pollard with a ratio of 40:60 for one weeks (feed adaptation). The rumen fluid samples were collected before the morning feeding. The fluid was carefully filtered through double layers of cheesecloth and transferred into pre-warmed (39°C) thermos bottles and was then transported to the laboratory within 30 min. The buffered inoculums were combination of rumen fluids and buffer solution with ratio 1:2 (v/v) for gas production analysis and 1:4 (v/v) for nutrient digestibility analysis under a continuous flux of CO₂.

In vitro gas production. Three hundred milligrams of substrate were weighed and placed in 100 mL glass syringe (Fortuna® model, Germany) for gas production analysis. Syringe was placed on a reciprocal shaker in a fan-driven incubator and pre warmed to 39°C for overnight. Each glass syringe filled with 30 mL of buffered inoculum and incubated for 48 h in an incubator at 39°C. At the end of incubations, gas production was recorded. Fermentation product was filtered and rumen fluid was measured for pH and protozoa cells. Others rumen fluid was centrifuged (3000 g, 10 min) to separate feed substrate from rumen fluid. Supernatant was tested for NH₃ concentration⁴⁰ and VFA profiles⁴¹. The supernatant was then centrifuged (10,000, 10 min) to separate microbial cells and the supernatant containing the enzyme. The precipitate was tested for microbial protein and the supernatant was tested for enzyme activity such as amylase, carboxymethyl cellulase, β -glucosidase⁴², and protease⁴³

In vitro rumen digestibility. The in vitro rumen digestibility analysis was carried out using the 2-stage in-vitro method³⁹. Five hundred milligram of substrate was weighed and placed in 100 mL serum bottle and incubated at 39°C for 48 h. After 48 h of incubation, the sample substrate was filtered to separate from rumen fluid. The substrate used analysis of in vitro rumen DM digestibility (IVDMD), in vitro OM digestibility (IVOMD), in vitro CP digestibility (IVCPD), and in vitro CF digestibility (IVCFD). In the second stage, the other samples were added with 6 mL of 20% HCL and 2 mL pepsin solution. The samples were incubated at 39°C for 48 h. At the end of incubation, the sample substrate was filtered to separate from rumen fluid and was analyzed for IVDMD, IVOMD, IVCPD, and IVCFD.

Statistical analysis. All experiment data were analyzed by one-way ANOVA. If there is a significant difference in values ($P < 0.05$) were continued with Duncan's new multiple range test (DMRT) to find out the difference in the mean value between each treatment. Data analysis uses the Statistical Product and Service Solution (SPSS) 16 application (IBM, USA).

Conclusions

The use of 200 μ L/L essential oil of nutmeg (*Myristica fragrans* Hoult.) was optimal level to reduced methane production without negatively affecting rumen fermentation, total nutrients digestibility (organic matter, dry matter, and crude fiber), and rumen enzymes activities (amylase, carboxymethyl cellulase, and β -Glucosidase). Furthermore, the addition of essential oil decreased protease enzyme activity and crude protein digestibility at 48 hours incubation which represents digestibility in the rumen. Total crude protein digestibility was enhanced at 96 hours of incubation, indicating improved digestion in the post rumen and adequate nutrition optimally for the host animal.

Declarations

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Author contributions statement

A.E.A. carried out the research, data analysis, and article drafting; D.S. conducted the research and data analysis; A.A.A. conducted the research and drafted the article; M.A.A. carried out the research, data analysis, and article drafting; M.A.A carried out the data analysis and article drafting; C.H. participated in data analysis; A.K. participated in manuscript finalization; M.M participated in manuscript finalization; and L.M.Y. as the project coordinator, organized the study design and finalized the manuscript. The authors declare no competing interests.

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Competing interests

The authors declare no competing interests.

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