# Substitution effects of rice for corn grain in total mixed ration on rumen fermentation characteristics and microbial community *in vitro*

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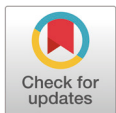
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Abstract

This study determined the substitution effects of rice for corn as the main grain source in a total mixed ration (TMR). *In vitro* rumen fermentation characteristics and microbes were assessed using two experimental diets. Diets included 33% dry matter (DM) of either corn (Corn TMR) or rice grains (Rice TMR). In a 48-h *in vitro* incubation, DM digestibility (IVDMD), neutral detergent fiber degradability (IVNDFD), crude protein digestibility (IVCPD), volatile fatty acids (VFAs), pH and ammonia nitrogen (NH_3-N) were estimated. Gas production has been calculated at 3, 6, 12, 24 and 48 h. Our results indicate that the gas production, VFAs, IVDMD, and IVNDFD of Rice TMR were higher than those of Corn TMR ($p < 0.05$). Ruminal pH and total fungi were significantly higher in Corn TMR ($p < 0.05$) than in Rice TMR; however, NH_3-N and IVCPD were not affected by treatment type. In conclusion, substituting rice for corn at 33% DM in TMR appears to have no negative effects on *in vitro* rumen fermentation characteristics. Therefore, rice grains are an appropriate alternative energy source in early fattening stage diets of beef cattle.

Keywords: Rice grains, Corn grains, Total mixed ration, *In vitro* fermentation

INTRODUCTION

The increasing import of agricultural feed, along with grain price volatility, origin, and nutritional value, is raising concerns about the ability of alternative energy sources to meet the increasing demands of Hanwoo beef production in Korea. Corn is an important feed source for domesticated animals, including farm animals, and an essential commodity for processing industrial bioethanol [1]. Approximately 99% of the corn used as feedstock in Korea is imported from the U.S. corn belt owing to its low cost and the region's production efficiency [2]. However, because of the rice cultivation reduction program initiated by the Korean government in 2015 to stabilize rice grain stockholding, rice is becoming more commonly used as a livestock feed in this country [3]. Korea is expected to use 450,000 MT of rice for

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feed in the 2019/2020 marketing year, with most of the combined rice stocks from previous years being reduced by the end of 2020 [4].

The characteristics of ruminal starch degradation vary depending on the type of cereal grain involved. Rice starch is digested more rapidly and has a higher effective degradability in the rumen than corn starch [5,6]. There are few studies evaluating the use of corn and rice in beef production. Despite the similar chemical composition and nutritive value of these grains, they exhibit different degradation characteristics in *in vitro* rumen fermentation systems with total mixed rations (TMRs) [7]. For example, in growing Hanwoo steer diets, as a 20% dry matter (DM) replacement of rice presented higher *in vitro* dry matter digestibility (IVDMD), *in vitro* crude protein digestibility (IVCPD), and volatile fatty acid (VFA) production than corn [8].

In dairy cow diets, 31% DM of rice substituted corn had a low impact on dry matter intake (DMI), milk yield and milk composition [9]. Nevertheless, in Korean early fattening stage steering diets for higher beef marbling, there are no findings regarding the use of rice as a corn replacement up to 33% DM. Rice as an alternative to corn for energy-rich diets can be essential for integrated crop and livestock production systems [10], balancing and boosting Hanwoo cattle productivity. Including energy-rich grains in fattening TMR diets can affect productivity and rumen fermentation characteristics [2]. Therefore, this study investigates the effects of substituting corn with rice as the main energy source at 33% DM in early fattening stage TMR diets on rumen fermentation characteristics and microbial populations.

MATERIALS AND METHODS

The protocols regarding the use of animal were closely examined and accepted by the Animal Research Ethics Committee of Pusan National University (Pusan, Korea, PNU-2019-2239).

Preparation of experimental diet and chemical analysis

The main ingredients of experimental TMR used in this study were timothy hay, alfalfa, rice, corn, and concentrate mixture (Nonghyup Feed Company, Miryang, Korea), and the dietary formulas along with chemical constituents of experimental TMR are described in Table 1. The experimental treatment consisted of two types of TMR: 1) The TMR containing 33% flaked corn (Corn TMR), 2) The TMR containing 33% rice grain (Rice TMR).

All the feed ingredients were air dried at 60℃ for 72 h and then milled using cyclone mill equipped with a 1 mm screen (Foss Tecator Cyclotec 1093, Foss, Hillerød, Denmark). The DM (#934.01), crude protein (CP; #976.05), acid detergent fiber (ADF; #973.18), ether extract (EE; #920.39), and ash (#942.05) were analyzed by the official methods of AOAC International [11]. According to the Kjeldahl process, total amount of nitrogen was calculated by a nitrogen combustion analyzer (Leco FP-528, Leco, St. Joseph, MI, USA) and multiplied by 6.25 to determine CP content. To determine the fiber content, lignin and neutral detergent fiber (aNDF) were evaluated according to the method described by Van Soest et al. [12]. The heat-stable form of α-amylase was utilized for estimation of aNDF and the volume of aNDF included residual ash. The chemical analysis for the Cornell Net Carbohydrate and Protein system (CNCPS) fraction analyzed with several modifications at Cumberland Valley Analytical Service (MD, USA) [13] and described in Table 2. Soluble protein (SOLP) was analyzed, and for each residue, the content of neutral detergent insoluble crude protein (NDICP) and acid detergent insoluble crude protein (ADICP) was also evaluated. The starch and ethanol-soluble carbohydrate (ESC) were measured. For carbohydrate fraction, A fraction (CA) was estimated to be same with ESC, and B1 fraction (CB1) represented starch. B2 fraction (CB2) was estimated as Non-fiber carbohydrate (NFC) − CA − CB1, and B3 fraction

Table 1. Chemical composition and dietary formulation of the experimental feeds

Items	Treatments[1]	
	Corn TMR	Rice TMR
Ingredients (% DM)		
Corn flake	33.0	0
Rice grain	0	33.0
Commercial concentrate mix	36.0	36.0
Alfalfa	7.0	7.0
Timothy	23.5	23.5
Vitamin and mineral mix[2]	0.5	0.5
Chemical composition (DM or as stated)		
DM (% as fed)	65.0	65.0
CP	14.9	14.8
aNDF	30.6	29.5
ADF	19.5	18.5
Lignin	4.45	4.17
Sugar	5.98	5.23
Starch	35.73	38.02
EE	3.50	3.19
Ash	5.22	5.42
TDN	72.4	73.8
NE_m (Mcal/kg of DM)	1.67	1.71

[1]Corn TMR, control diet containing 33% flaked corn; Rice TMR, the diet containing 33% rice grain.

[2]Vitamin A, 33,330,000 IU/kg; vitamin D, 40,000,000 IU/kg; vitamin E, 20.86 IU/kg; Cu, 20 mg/kg; Mn, 90 mg/kg; Zn, 100 mg/kg; Fe, 250 mg/kg; I, 0.4 mg/kg; Se, 0.4 mg/kg.

TMR, total mixed ration; DM, dry matter; CP, crude protein; aNDF, neutral detergent fiber analyzed with heat-stable α-amylase; ADF, acid detergent fiber; EE, ether extract; TDN, total digestible nutrients; NE_m, net energy for maintenance.

(CB3) was calculated with the following equation (aNDF – NDICP) – (2.4 × Acid detergent lignin [ADL]). C fraction (CC) was evaluated as 2.4 × ADL. For protein fraction, A and B1 fractions (PA + PB1) was equal to SOLP, and B2 fraction was estimated according to the equation 100 – NDICP – SOLP, and B3 fraction was calculated by the equation NDICP – ADICP. C fraction of protein (PC) was equal to ADICP. To estimate the total digestible nutrient (TDN) and net energy for maintenance (NE_m), several equations in the NRC were used [14,15]. NFC was calculated as follows [15]:

$$100 - ash - EE - CP - (aNDF - NDICP)$$

In vitro fermentation

Rumen fluid has been used for the *in vitro* incubation and collected from two cannulated Holstein cattle (body weight [BW] 650 ± 30 kg), before they were fed morning feed at the Center for Agriculture Research of Pusan National University (Miryang, Korea). The cannulated cattle were completely adapted to a diet comprising 400 g/kg of a commercial concentrate mix and about 600 g/kg of timothy hay. Collection of rumen fluid was done in a thermos bottle and then immediately transferred to the laboratory within 30 minutes. Eight layers of cheesecloth were used to filter rumen fluid and then thoroughly mixed with three times volume of *in vitro* buffer solution under strict anaerobic condition [16]. Each ground experimental substrate (0.5 g) was placed into the nylon bags (R510, Ankom Technology, NY, USA) and were subsequently sealed and transferred into

Table 2. Carbohydrate (%CHO) and protein (%CP) fractions of the experimental feeds (%DM)

Items	Treatments[1]	
	Corn TMR	Rice TMR
Carbohydrate fraction (%CHO)[2]		
CA	7.83	6.83
CB1	46.78	49.67
CB2	8.24	7.94
CB3	23.04	22.48
CC	13.99	13.07
Protein fraction (%CP)[3]		
PA + PB1	23.84	23.67
PB2	61.24	61.54
PB3	5.86	5.92
PC	10.70	10.38

[1]Corn TMR, control diet containing 33% flaked corn; Rice TMR, the diet containing 33% rice grain.

[2]CA, carbohydrate A fraction; CB1, carbohydrate B1 fraction; CB2, carbohydrate B2 fraction; CB3, carbohydrate B3 fraction; CC, carbohydrate C fraction.

[3]PA + PB1, protein A and B1 fraction; PB2, protein B2 fraction; PB3, protein B3 fraction; PC, protein C fraction.

TMR, total mixed ration; DM, dry matter; CHO, carbohydrate; CP, crude protein.

250 mL serum bottles. Each treatment had four replicates and four bags were used for each bottle. In each bottle, 140 mL of mixture containing rumen fluid and *in vitro* buffer solution were put with continuous flushing of O_2-free CO_2 gas. The bottles were capped with butyl rubber caps and incubated at 20 rpm for 48 h and 39℃ on a rotary incubator (JSSI-300 T, JS Research, Gongju, Korea).

After 48-h incubation, the content of IVDMD, *in vitro* aNDF degradability (IVNDFD), IVCPD, pH, VFA, and ammonia nitrogen (NH_3-N) were analyzed. The gas production at 3, 6, 12, 24, and 48 h was estimated by a pressure transducer (Sun Bee Instrument, Seoul, Korea) [17]. The gas parameters were measured using a simple exponential model following equation [18]:

$$V_T = 0 \qquad\qquad (0 \leq T \leq L)$$
$$V_T = V_{max} \times \{1 - e^{[-kg \times (T-L)]}\} \qquad\qquad (T \geq L)$$

The T is time (h), L is lag time (h), and e is the exponential function. The value of K_g is the fractional rate of gas production (h^{-1}). The V_{max} is the theoretical maximum production of gas (mL), and V_T is volume of gas at time T (mL). After 48-h of incubation, the bottle caps were removed transferred into ice to stop microbial fermentation. Subsequently, bags were removed from the incubated bottles and rinsed with tap water until it was clear. All the sealed bags were weighed for the estimation of IVDMD after oven drying at 60℃ for 72 h. For the assessment of IVNDFD, the neutral detergent fiber (NDF) content of each weighted bag was assessed [19]. Centrifugation of the sample fluid (1.8 mL) at 20.000×g, at 4℃ for 20 minutes, and then removal of the supernatant. The remaining pellet was stored at −80℃ until extracting microbial DNA. The rumen fluid pH was measured using the pH meter (FP20, Mettler Toledo, OH, USA) and the remaining rumen fluid was centrifuged at 15.000×g, at 4℃, for 10 min, and stored at −20℃ for analysis of VFA and NH_3-N. Before carrying out the VFA and NH_3-N analysis, the stored rumen fluid was melted and centrifuged for 15 min at 20,000×g. The supernatant (200 μL) was diluted with 800 μL of ethyl alcohol (4023-2304, Daejung Chemicals, Siheung, Korea) and the diluted mixture were analyzed on VFA using a gas chromatography (Agilent 7890A, Agilent Technology, CA, USA) mounted with a flame ionization detector and Nukol™ Fused silica capillary column (30 m × 250 μm ×

0.25 μm; Supelco, PA, USA). The temperature (oven, injector, detector) was set at 90, 90–200, and 230℃, respectively, and the nitrogen was used as a carrier gas (flow rate, 30 mL/min). The NH_3-N concentration was measured by the described method of Chaney and Marbach [20], with some adjustments. Briefly, the supernatant of centrifuged rumen fluid (2 μL) was mixed with phenol color reagent (100 μL, 0.25 g of sodium nitroferricyanide, 50 g of phenol, 1 L distilled water) and alkali hypochlorite (100 μL, 16.8 mL of sodium hypochlorite, 25 g of sodium hydroxide, 1L distilled water). The mixture was then incubated at 37℃ for 15 minutes and the concentration of NH_3-N was analyzed by the absorbance at an optical density (630 nm) using a microplate reader (iMARK, Bio-Rad, CA, USA).

Microbial genomic DNA extraction and quantitative polymerase chain reaction

The genomic DNA from pellet was extracted by the method described by Yu and Mosrrison [21]. The DNA concentration and purity were evaluated with a NanoDrop (ND-1000, Thermo Fisher, MA, USA). Quantitative polymerase chain reaction (qPCR) was carried out using a CFX 96 Touch system (Bio-Rad Laboratories Inc., CA, USA). The primer set used in this study are represented in Table 3 [22,23]. Each reaction mixture volume was 20 μL containing 10× buffer (2 μL, BioFACT, Daejeon, Korea), 10 mM dNTP mixture (0.5 μL, BioFACT, Daejeon, Korea), 10-fold diluted genomic DNA (1 μL), 10 μM primer-set (each 1 μL), 1 μL reverse (10 μM), taq polymerase (0.1 μL, BioFACT, Daejeon, Korea), Evagreen (1 μL, SolGent, Daejeon, Korea), and bio-grade water (13.4 μL). All the reactions were conducted in triplicate, the procedure condition of qPCR was as follows: initial denaturation of DNA at 95℃ for 10 min, and 40 cycles of denaturation at 95℃ for 30 s, annealing at 60℃ for 30 s, and elongation at 72℃ for 30 s, followed by a final elongation at 72℃ for 5 min. At the end of extension stage, fluorescence was noted. All the amplicon specificity was checked through dissociation curve by rising the temperature, at a rate of 1℃ per 30 s, from 60℃ to 95℃. A standard plasmid having respective target sequence was used for absolute quantification of each microbe. The copy number of each standard primer was calculated as described by [24] and diluted with 10-fold serial dilution. CFX manager software (Bio-Rad, CA, USA) was used to compare the quantification of each microbe with the standard curve.

Statistical analysis

Statistical analysis was done by the PROC TTEST procedure of SAS 9.3 (SAS Institute, NC, USA), and the statistical significance was defined at $p < 0.05$. The statistical trend was represented at $0.05 \le p < 0.1$.

Table 3. Primers used for quantitative PCR

Target species	Primer	Sequence (5' → 3')	Size (bp)	Efficiency[1]	References
General bacteria	F	CGGCAACGAGCGCAACCC	130	1.89	[11]
	R	CCATTGTAGCACGTGTGTAGCC			
Protozoa	F	GCTTTCGWTGGTAGTGTATT	223	1.92	[14]
	R	CTTGCCCTCYAATCGTWCT			
Fungi	F	GAGGAAGTAAAAGTCGTAACAAGGTTTC	120	2.06	[11]
	R	CAAATTCACAAAGGGTAGGATGATT			

[1]Efficiency is calculated as $[10^{-1/slope}]$.

PCR, polymerase chain reaction; bp, base pair.

RESULTS

The Rice TMR effects on gas production and parameters in comparison with those of Corn TMR are presented in Table 4. *In vitro* gas production was significantly higher in Rice TMR than in Corn TMR at all observed time points ($p < 0.05$). In the fitted gas parameters, V_{max} was also significantly higher in Rice TMR ($p < 0.01$) than in Corn TMR, whereas no significant difference in K_g was detected. Ruminal pH was higher in the Corn TMR diet ($p < 0.05$) than in the Rice TMR diet; however, no difference in the NH_3-N concentration was detected between the two TMR diets ($p = 0.44$). Total VFA production was significantly higher in Rice TMR ($p < 0.01$) than in Corn TMR; however, regarding individual VFAs, there were no significant differences in the acetate, propionate, and butyrate concentrations ($p = 0.17$, $p = 0.63$, and $p = 0.39$, respectively). The ratio of acetate to propionate was therefore no different ($p = 0.45$). Regarding ruminal digestibility indices, IVDMD and IVNDFD were higher in Rice TMR than in Corn TMR ($p < 0.01$ for both), whereas no difference was detected for IVCPD ($p = 0.24$; Table 5). In the ruminal microbial community,

Table 4. *In vitro* gas production characteristics of experimental diets incubated in buffered rumen fluid

Items	n	Treatments[1]		SEM	*p*-value
		Corn TMR	Rice TMR		
Gas (mL/g DM)					
3 h	4	19.5	21.6	0.66	< 0.05
6 h	4	47.6	51.2	1.48	< 0.05
12 h	4	103.6	113.3	2.18	< 0.01
24 h	4	174.0	187.7	1.74	< 0.01
48 h	4	225.3	241.4	2.36	< 0.01
Fitted parameters of gas[2]					
V_{max}	4	269.6	286.2	4.06	< 0.01
K_g	4	0.039	0.047	0.0011	0.21

[1]Corn TMR, control diet containing 33% flaked corn; Rice TMR, the diet containing 33% rice gain.

[2]V_{max}, theoretical maximum gas production (mL/g DM); K_g, fractional rate of gas production (h^{-1}).

TMR, total mixed ration; DM, dry matter; SEM, standard error of the mean.

Table 5. Fermentation characteristics of experimental diets incubated in buffered rumen fluid

Items	n	Treatments[1]		SEM	*p*-value
		Corn TMR	Rice TMR		
pH	4	6.2	6.1	0.02	< 0.05
NH_3-N (mg/100 mL)	4	35.3	33.2	2.47	0.44
Total VFA (mM)	4	84.6	91.5	1.71	< 0.01
Acetate (mmoL/moL)	4	544.2	551.3	4.52	0.17
Propionate (mmoL/moL)	4	276.3	274.3	3.99	0.63
Butyrate (mmoL/moL)	4	131.5	129.7	1.90	0.39
A:P ratio	4	1.98	2.01	0.044	0.45
IVDMD (%)	4	82.9	87.0	1.01	< 0.01
IVNDFD (% aNDF)	4	64.7	70.0	1.14	< 0.01
IVCPD (% CP)	4	85.1	88.3	2.49	0.24

[1]Corn TMR, control diet containing 33% flaked corn; Rice TMR, the diet containing 33% rice gain.

TMR, total mixed ration; SEM, standard error of the mean; NH_3-N, ammonia nitrogen; VFA, volatile fatty acid; A:P ratio, acetate to propionate ratio; IVDMD, *in vitro* dry matter degradability; IVNDFD, *in vitro* neutral detergent fiber degradability; aNDF, neutral detergent fiber analyzed with heat-stable α-amylase; IVCPD, *in vitro* crude protein degradability; CP, crude protein.

the substitution of rice for corn tended to increase the total protozoal count (p = 0.08) in Rice TMR, and significantly increase the total fungal count (p < 0.01) in Corn TMR. However, the total bacterial count was not affected by treatment type (p = 0.57; Table 6).

DISCUSSION

High-energy grain sources are commonly used to enhance growth efficiency in early fattening diets for beef cattle. The availability of a grain source varies with grain type and processing methods. The use of high-energy grain sources with a faster digestion rate will induce ruminal acidosis in the transition period from a high forage to a high grain diet [25]. Thus, it is important to select dietary energy sources that are appropriate to meet nutritional requirements while maintaining a healthy rumen environment. Yang et al. [7] reported that the use of rice grain in TMR at 70% DM can lead to higher IVDMD than the use of corn and wheat grain. Replacing corn with rice at 20% DM leads to higher IVDMD and VFA production without significant change to ruminal pH [8], suggesting that there is potential to replace conventional grains in fattening TMR diets.

In the present study, it was observed that the replacement of rice for corn at 33% DM in TMR increased not only gas emissions at all observed time points but also V_{max}. The amount of gas emitted is calculated during *in vitro* ruminal fermentation to predict the DM degradability of cereal grains used as ruminant feed [26]. However, protein is another essential nutrient for energy-rich TMR ruminant diets, providing an efficient supply of amino acids that serve as building blocks for body tissues in growing beef cattle [27]. In this study, NH_3-N concentration was not different between the treatments (p = 0.44). Furthermore, during the *in vitro* fermentation of Rice and Corn TMR, IVCPD was more than 80% at 48 h of incubation without any significant differences (p = 0.24). These findings are close to those of previous studies in which the substitution of rice grain at 70% and 20% DM resulted in higher IVCPD, whereas the concentration of NH_3-N between rice and corn TMR diets was not different [7,8].

Cereal grains are important food sources, supplying energy reserves to sustain the dietary needs of growing beef and dairy cattle [28]. Microbial fermentation results in VFAs as the final products of a ruminant's diet and provides 70% of the supplied energy; the conversion of propionate to glucose is beneficial to the body's growth and maintenance requirements [29]. In this study, Rice TMR had higher total concentrations of VFA, without any significant differences between the treatments regarding the individual VFA proportions and the acetate to propionate ratio. In a recent *in vitro* analysis, 20% DM substitution of rice for corn in a TMR diet increased the total VFA while retaining a similar ratio of acetate to propionate [8]. Contrary to our findings, Oh et al. [30] reported decreased propionate and increased acetate in Hanwoo steer diets with 50% DM of rice grains. However, compared to those with corn, lower acetate and higher propionate concentrations were

Table 6. Rumen microbial population of experimental diets incubated in buffered rumen fluid

| Items | n | Treatments[1] | | SEM | *p*-value |
		Corn TMR	Rice TMR		
Organism[2]					
General bacteria	4	7.81	7.54	0.475	0.57
Protozoa	4	7.33	7.98	0.363	0.08
Fungi	4	1.96	1.54	0.095	< 0.01

[1]Corn TMR, control diet containing 33% flaked corn; Rice TMR, the diet containing 33% rice gain.

[2]General bacteria, × 10^{10} copies/mL of rumen fluid; Protozoa, × 10^9 copies/mL of rumen fluid; Fungi, × 10^7 copies/mL of rumen fluid.

TMR, total mixed ration; SEM, standard error of the mean.

identified with 40% DM of brown rice [5].

Heat-treated corn flakes are beneficial for ruminants, in that they provide a slow release of easily digested nutrients [31]. In addition, heat-treated cereal grains in the rumen may have improved digestibility owing to starch gelatinization [32]. However, our results show that an untreated rice grain TMR diet had higher IVDMD than a heat-treated corn flake TMR diet. Generally, NDF in the cereal grains is not particularly effective in stimulating rumination [33]. However, IVNDFD was significantly higher for Rice TMR during a 48-h *in vitro* incubation, despite the similarity of diet composition and nutrient values between the two TMR diets. In their comparison of steer diets, Oh et al. [30] recorded low fiber digestibility at 50 and 100% DM substitution of rice for corn. Likewise, replacing corn with rice at 40% DM at increasing levels in a lactating cattle diet decreased NDF digestibility [5].

Dietary carbon sources can be converted to energy via nutrient fermentation pathways of the rumen microbial biomass; bacteria, protozoa, and fungi are the three kingdoms that account for most microbial biomass [34]. Regarding the microbial community in the rumen, the substitution of rice grains tended to increase the protozoa (p = 0.08) and lead to a higher IVNDFD. However, total fungi were significantly higher for Corn TMR, and total bacteria did not differ between the treatments (p = 0.57). This may relate to the fact that fungi are anaerobic obligates that produce cellulases and xylanases. This allows them to more effectively derive nutrition from the fermentation of carbohydrates [35] in ruminants with diets containing comparatively high levels of cereal grains [33]. Substituting rice for corn at 33% DM led to higher gas production, VFA, IVDMD, and IVNDFD. There was no significant difference between our two treatments regarding the number of ruminal bacteria present. However, the protozoal biomass tended to be greater in Rice TMR, whereas the fungi biomass was significantly greater in Corn TMR. The substitution of rice grains in the TMRs at a level of 33% DM had no negative effects on rumen fermentation characteristics, and such diets can be used to grow Hanwoo steers. Further *in vivo* research studies will be helpful to evaluate the various growth performance parameters and physiological indicators for beef cattle during the finishing period in Korea.

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